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The role of integrins and neuregulins in axoglial interaction in central nervous system myelination

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Declaration

I hereby declare that the studies undertaken for the completion of this thesis represent work performed by the author, except where acknowledged by reference. These have not been previously submitted for another degree or qualification.

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Abstract

Oligodendrocytes in the central nervous system (CNS) are responsible for wrapping axons with myelin in order to insulate them and allow for a faster conduction of the nervous impulse. The axonal signals that determine whether an axon is myelinated, and what regulates the number of wraps is still not fully understood. The importance of signals that initiate myelination is significant because they may point to novel therapies for Multiple Sclerosis, where remyelination prevents the axon degeneration that is thought to underlie chronic disease.

Neuregulin 1 (Nrg1) has been identified as a key axonal signaling molecule that regulates myelin thickness and glial fate in the peripheral nervous system (PNS). In the PNS, neuregulin I type III is a necessary and sufficient signal that regulates axoglial interaction. The role of neuregulin in the CNS remains unclear and controversial.

Integrins, the major family of extracellular matrix (ECM) receptors are involved in the regulation of many fundamental cellular functions. Interaction with a wide range of receptors including growth factor receptors is well described. Our lab showed that α6β1 integrin regulates oligodendrocyte survival signaling by amplification of neuregulin activity.

We have found that mice expressing a dominant-negative β1 integrin (that reduces β1 integrin signaling independently of ligand binding) in myelinating oligodendrocytes require a larger axon diameter to initiate myelination. These results suggest that there are other signals in the axon that also contribute to initiation of myelination. We therefore hypothesized that β1 integrin and neuregulin act in concert and play a role in axoglial interactions that sense the axon size and initiate myelination. By crossing the dominant negative β1 integrin mice with heterozygous mice for neuregulin 1 and analyzing myelination, we have found that neuregulin does not enhance the phenotype previously described. This result together with previous reports that mice lacking NRG1, ErbB3 or

ErbB4 (the neuregulin receptors expressed on oligodendrocytes) have normal CNS myelin sheaths demonstrates that neuregulin 1 is not required for CNS myelination.

Interestingly, neuregulin 1 has been associated as a susceptibility gene in schizophrenia, a disease independently associated with myelin abnormalities (Davis *et al.*, 2003; Hakak *et al.*, 2001). Post-mortem brains of schizophrenic patients showed an increased level of neuregulin 1 type IV. We have analysed mice overexpressing neuregulin 1 type IV (Nrg1 type IV) and show that increased levels of neuregulin 1 type IV does not alter the brain morphology or myelin pattern and integrity. A possible explanation is that since neuregulin 1 type IV is human specific, the mice lack species-specific receptors or other neuregulins have compensatory equilibrium mechanism that are not destabilized by overexpression of neuregulin 1 type IV.

Abbreviations

Aβ	amyloid-β peptides
AChR	acetylcholine receptor
Akt	serine/thereonine kinase
APP	β-amyloid precursor protein
ATP	adenoside triphosphate
βTD	β-tail domain
Bace1	beta-site amyloid precursor protein-cleaving enzyme 1
BBB	blood-brain barrier
BDNF	brain-derived neurotrophin factor
BnTX	botulinum toxin A
CGT	UDT-galactose ceramide galactosyltransferase
CIB	calcium integrin binding protein
CMT	Charcot-Marie-Tooth disease
CNP	2',3'-cyclic nucleotide 3'-phosphodiesterase
CNS	central nervous system
CRD	cystein rich domain
DMEM	Dulbecco's modified Eagle medium
DRG	dorsal root ganglion
Dnβ3	dominant negative β3 integrin
E(#)	embryonic day #
EAE	experimental allergic encephamomyelitis
EAN	experimental allergic neuritis
ECM	extracellular matrix
EGF	epidermal growth factor

EM	electron microscopic
ERK	extracellular signal regulated kinase
FAK	focal adhesion kinase
FAT	focal adhesion targeting
FCS	fetal bovine serum
Fdu	fluorodeoxyuridine
FGF	fibroblast growth factor
GalC	galactocerebroside
GAP	GTPase-activating proteins
GBS	Guillain-Barré syndrome
GDI	guanine nucleotide dissociation inhibitors
GEF	guanine nucleotide exchange factor
GGF	glial growth factor
GlcC	glucocerebroside
Grb2	growth-factor-receptor-bound-2
HB-EGF	heparin-binding EGF
ILK	integrin-linked kinase
JNK	Jun-amino terminal kinase
MAG	myelin-associated glycoprotein
MAPK	mitogen-activated protein kinase
MBP	myelin basic protein
MDL	major dense lines
MIDAS	metal-ion-dependent adhesion site
MOG	myelin oligodendrocyte glycoprotein
MS	multiple sclerosis
Na^+	sodium
NCAM	neural cell adhesion molecule
NDF	Neu differentiation factor

NF-κB	nucleofactor κB
NGF	nerve growth factor
NMJ	neuromuscular junction
N-WASP	Neural Wiskott-Aldrich syndrome protein
OCS	organotypic cerebellar slice cultures
OPC	oligodendrocyte precursor cell
P(#)	postnatal day #
P0	Ig-CAM myelin protein zero
PDGF	platelet-derived growth factor
PFA	paraformaldehyde
PI3K	phosphatidylinositol-3-OH kinase
PIP2	phosphatidyl inositol biphosphate
PKC	protein kinase C
PLP	tetraspan proteolipid protein
PMD	Pelizaeus-Merzbacher disease
PNS	peripheral nervous system
Prx	periaxin
PSI	plexin-semaphorin-integrin
PTB	phosphotyrosine binding
RT	room temperature
SC	Schwann cells
SH	Src-homology domain
SMDF	sensory and motor neuron derived factor
SOS	son-of-sevenless
TACE	tumour necrosis factor-α converting enzyme
TH	thyroid hormone
Trk	tropomyosin receptor kinase
TTX	tetrodotoxin
Y#	tyrosine residue #

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Chapter 1

Literature review

Chapter 1

Literature review

1. Introduction

The overall aim of this PhD thesis is to understand the role of integrins and neuregulins in the process of myelination in the central nervous system (CNS).

The signals that determine whether an axon is myelinated and that govern the number of wraps around the axon are poorly understood. All axons over a critical diameter become myelinated, with the threshold axon diameter for myelination being 0.2 µ.oipm in CNS and 1 µm in the peripheral nervous system (PNS) (Waxman and Bennet, 1972; Voyvodic, 1989). The thickness of the sheath formed is linked to the axon diameter – the so called g-ratio (Friede, 1972). In the PNS, neuregulin 1 type III has been identified as a necessary and sufficient signal to regulate myelination (Taveggia *et al.,* 2008). Such an analogue thus far has not been described for CNS. Integrins, a major family of extracellular matrix (ECM) receptors, are involved in the regulation of many cellular processes and previous work from our lab showed that α6β1 integrin regulates oligodendrocyte survival signalling through amplification of neuregulin activity (Colognato *et al*, 2002). Therefore we hypothesized that β1 integrin and neuregulin 1 may have a cooperative role in CNS myelination.

The introduction of this thesis will review the current knowledge related to myelin and the myelination process. Subsequent chapters will discuss work carried out in relation to integrins, neuregulins and schizophrenia and relevant literature will be discussed in each chapter.

1.1 Organization of the nervous system

During evolution, animals adapted in order to better cope with their environment. One of the most amazing evolutionary adaptations is related to the development of the nervous system. The nervous system is responsible for gathering information from the environment, processing this information, and attributing a specific signal that will lead to an adequate response. In lower organisms, the nervous system is very simple, but becomes increasingly complex in higher organisms.

One of the most important features of the nervous system is to conduct information in the form of a nervous impulse. This has to be both efficient and fast; therefore the nervous system could evolve in two distinct ways to achieve this, either by increasing the dimensions of the axon, or by insulating the axons with a lipidic membrane – myelin – to allow rapid conduction of the nervous impulse. Conduction velocity is directly proportional to the axon diameter in unmyelinated axons. By increasing the axon diameter the conduction is raised due to the lower resistance registered in the fibres. This type of evolutionary mechanism can be found in squid (with its famous giant axon), fruit flies and copepodes. One of the setbacks for this type of mechanism is the correlation between the size of the animal and the size of the axon. Bigger animals would need a substantial increase in their axon diameter in order to improve performance. The second strategy allows a faster propagation of the impulse by insulating the axon with myelin which increases the resistance and decreases the electrical capacitance due to its non-conducting properties (reviewed by Allen and Barres, 2009; Colman, 2007). This strategy allowed these organisms to increase their size, without the need to increase their cranium, enabling a fast reactive response, giving these organisms a complex but compact nervous system, a reduction of energy requirements (the squid giant axon requires 5000 times more energy than a 12 μm myelinated axon of a frog) and correct propagation of the nervous impulse (Hartline, 2008; Pedraza *et al.*, 2001; Quarles. 2006).

Vertebrates possess a highly demanding and intricate nervous system that comprises peripheral nervous system (PNS), which consists of the peripheral nerves and their ganglia, and central nervous system (CNS) comprising the brain and spinal cord (Stevens *et al.,* 1997). Both systems share the same type of cells: neurons and glia. Neurons are responsible for gathering information from sensory receptors, processing that information and generating appropriate signals to effector cells by transmitting electrical signals in the form of action potentials (Stevens *et al.,* 1997). Neurons have three distinct areas: the soma or cell body, axon and dendrites. Glial cells in the CNS were first described by Richard Virchow who named them "nervenkitt", which means "nerve glue" and were once thought to be the brain's connective tissue, helping with support functions (reviewed by Somjen, 1988).

In more recent years glial cells have been more thoroughly described and it is now established that they have a more defined role as regulators of CNS development and function (reviewed by Allen and Barres, 2009). Glial cells outnumber neurons and are a large part of the nervous tissue and their proportion increases with the degree of evolution, from 25% in fruit flies to 90% in the human brain (reviewed by Pfrieger and Barres, 1995). The CNS comprises microglia and macroglia (oligodendrocytes and astrocytes), while in PNS there are Schwann cells, enteric glia and satellite cells (Jessen, 2004).

The glial cells in the CNS are derived from neural precursor cells of the ventricular zone (Jessen, 2004). Microglia are the resident immune cells of the CNS, they survey the brain for damage and infection and act as immune regulators by secreting cytokines/growth factors and act as macrophages by engulfing dead cells and debris (Ruckh *et at.,* 2012). Astrocytes outnumber oligodendrocytes, with a star like appearance and many processes radiating in all directions. These processes control axonal outgrowth and maintain blood-brain barrier (BBB) integrity which is crucial for CNS protection against foreign substances; they are also involved in embryogenesis, fluid transport, energy metabolism, neurotransmitter homeostasis and synaptogenesis (by removing excess neurotransmitter molecules from the extracellular space and helping the accurate encoding of synaptic signals and

neurotransmission) and structural support (Allen and Barres, 2009; Jessen, 2004; Purves *et al.,* 2001; Stevens *et al.,* 1997). Oligodendrocytes were first characterized in the 1920s by Rio Hortega, using silver impregnation (Hortega, 1928). Work by Wilder Penfield confirmed the identity of oligodendrocytes, and further suggested that they were the myelin forming cells of the CNS (Penfield, 1924). Oligodendrocytes extend several processes that contact and form distinct myelin internodes, sometimes on different axons (a single oligodendrocyte can myelinate up to 30-40 axons) (Jessen, 2004). Myelination occurs discontinuously along the axon forming the internodes, leaving unmyelinated gaps, known as nodes of Ranvier. This allows for membrane depolarization to be located specifically in the nodes, termed saltatory conduction, by enabling current flow across the axolemma to the nodes of Ranvier which have a high concentration of voltage-dependent sodium channels (Sherman and Brophy, 2005).

Glial cells in the PNS derive from the neural crest (Dulac *et al.,* 1991). The enteric glia, found in the ganglia of the gut, are structurally and biochemically similar to astrocytes and are involved in the synaptic and muscular digestive processes (reviewed by Sofroniew and Vinters, 2010). Satellite glial cells are small support cells that surround neurons in ganglia to which they supply nutrients (Jessen, 2004; Stevens *et al.,* 1997). Schwann cells can either be myelinating or non-myelinating. The non-myelinating Schwann cells play a role in metabolic and mechanical support, while the myelinating Schwann cells are analogous to oligodendrocytes, wrapping axons with myelin sheaths (Sherman and Brophy, 2005).

1.2 Glial cells and myelination

Oligodendrocytes form one of the most highly specialised cellular structures in the body, the myelin sheath. They ensheath axons with myelin, in order to form electrical insulation around nerve fibres, allowing axon potentials (the electrical impulses propagated along them), to be transmitted faster in a process known as

saltatory conduction. During saltatory conduction, axon potentials leap among specialised gaps in the myelin sheath, called nodes of Ranvier where sodium channels are clustered (Baumann *et al.*, 2001; Jessen, 2004)

Myelination is a highly demanding process, with myelin being one of the most specialised cellular structures in the body. An oligodendrocyte in the rat brain, during the active process of myelination, has to produce approximately 5000 μm^2 of myelin surface daily, and approximately 10^5 myelin proteins per minute (Jessen, 2004; Pfeiffer *et al.*, 1993).

1.2.1 PNS vs CNS myelination

In this next section the differences between PNS and CNS myelination will be reviewed. As mentioned previously Schwann cells are responsible for PNS myelination, whilst in the CNS this function is mediated by oligodendrocytes. Whereas a single oligodendrocyte in the CNS can myelinate multiple axons and extend up to 40 different processes, in the PNS, Schwann cells myelinate in a 1:1, Schwann cell:axon ratio (Fig. 1.1) (Sherman and Brophy, 2005).

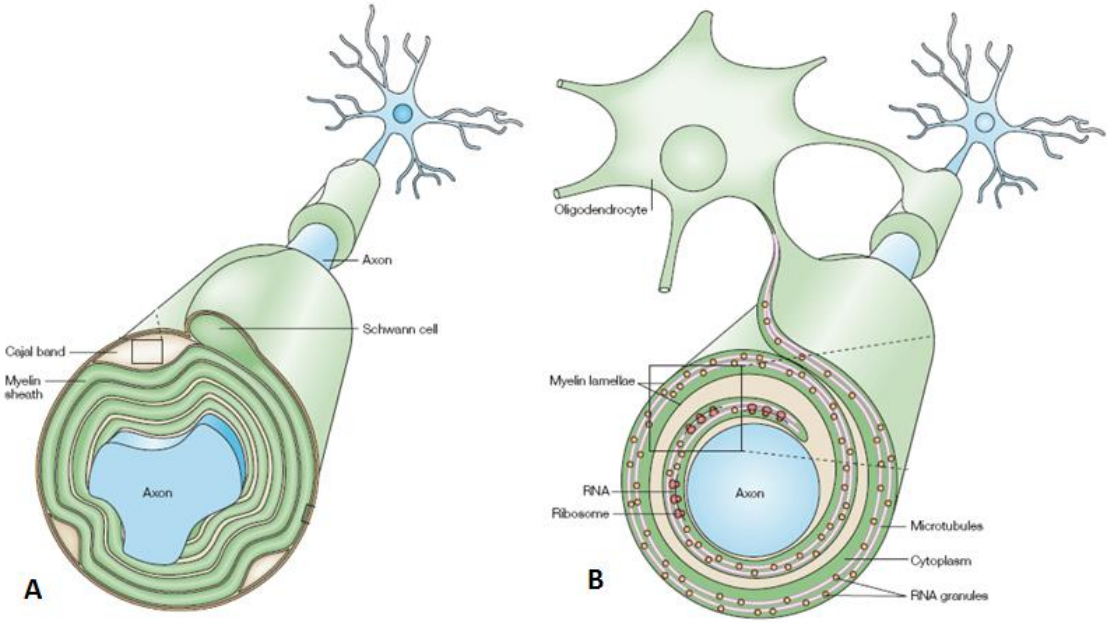


Figure 1.1 – Myelination in the peripheral and central nervous system. Glial cells are responsible for wrapping myelin along the axon to provide insulation and increase the propagation of the nervous impulse. In the peripheral nervous system Schwann cells (A) myelinate in a 1:1, cell:axon ratio while in the central nervous system oligodendrocytes (B) are capable of extending multiple processes and interact with different axons. Reproduced with minor changes from Sherman and Brophy, 2005.

In the PNS, Schwann cells arise from the neural crest and can be myelinating or non-myelinating. This distinction is based on the calibre of the axon they initially associate with. If a Schwann cell establishes contact with a large diameter axon it will differentiate and become a myelinating cell, whilst those in contact with small diameter axons maintain their non-myelinating phenotype. These non-myelinating Schwann cells form the Remak bundle by surrounding multiple small diameter axons. The number of axons engulfed by non-myelinating Schwann cells varies depending on location, with large numbers of axons observed in these bundles in L5 dorsal root ganglion (DRG) (in 50% of cases these bundles present more than 20 axons), and small numbers in the distal nerve segments (with an average of 3 axons) (Murinson and Griffin, 2004). Transgenic mice in which these bundles were perturbed showed progressive axonal degeneration and neuropathy (Chen *et al.*, 2003), revealing the role of non-myelinating Schwann cells in axonal integrity maintenance (Nave and Trapp, 2008). Myelinating Schwann cells surround a group of nerves and in a process termed radial sorting, sort axons to the periphery of the bundle establishing contact and extending myelin over the internode (Sherman and Brophy, 2005).

Myelination in the PNS and CNS is regulated by different signals, and although myelin shares its main components across both systems, there are differences in the proteins expressed by PNS and CNS.

A comparison of myelin proteome from CNS and PNS showed a high percentage of proteins are shared between both systems (Monasterio-Schrader *et al.*, 2012). Myelin basic protein (MBP) is one such protein that is highly abundant and involved in myelin compaction. However, there are myelin proteins expressed selectively in PNS

or CNS; PNS myelin has P0 (Ig-CAM myelin protein zero) as one of its main constituents, whilst in the CNS this protein is not present, being replaced by the tetraspan proteolipid protein (PLP) (Schliess and Stoffel, 1991). There are other examples of exclusive proteins such as claudin 11 (Bronstein *et al.*, 2000) in the CNS and periaxin (Prx) in the PNS (reviewed by Kursula, 2008).

Whereas myelin proteins can differ in the PNS and CNS, there are signals that can modulate both systems, such as beta-site amyloid precursor protein-cleaving enzyme 1 (Bace1). Bace1 is a protease responsible for cleaving the β-amyloid precursor protein (APP) into amyloid-β peptides (Aβ), which aggregate in brains of Alzheimer's disease patients (Sisodia and George-Hylop, 2002). Bace1 is commonly expressed in neurons and modulates APP processing through Nogo (a component of myelin) (Woolf, 2003). Bace1 null-mice show hypomyelination from early stages of development and reduced myelin thickness, with a reduction of compact myelin protein levels (MBP and PLP), but no alteration of uncompacted myelin proteins (MAG and MOG) both in PNS and CNS, which leads to impaired neurological function such as reduced grip strength and a lower pain threshold . Axonal secreted neuregulin 1 is cleaved by Bace1, since higher levels of full-length neuregulin 1 type III, and not the cleaved and potentially active form, were found in these mutant mice. Neuregulin 1 activates the cell surface receptor ErbB which leads to activation of phosphatidylinositol-3-OH kinase (PI3K) and Akt. A possible mechanism that could explain these observations would be that neuregulin 1 cleavage by Bace1 is necessary for serine/thereonine kinase (Akt) phosphorylation (since Akt phosphorylated levels were also decreased in these mice) (Hu *et al.*, 2006). Transgenic mice that overexpress a constitutively active Akt in oligodendrocytes have increased numbers of myelin sheaths wrapped around the axon (therefore enhancing myelination) but show no alteration in oligodendrocyte development (i.e., proliferation or survival). Together, these observations suggest that impairment in this signalling pathway leads to abnormal myelination both in CNS and PNS (Narayanan *et al.*, 2009).

Signals that regulate axoglial interaction and myelination have long been investigated. In the PNS, neuregulin 1 type III has been identified as one of these regulators with the capacity of solely affecting myelination and myelin thickness. The levels of axonal neuregulin 1 type III in the PNS determines both the decision for Schwann cells to myelinate and the number of myelin wraps around the axon. Overexpression of neuregulin 1 type III *in vitro* promotes myelination of axons that would normally be unmyelinated; while transgenic mice with increased levels of neuregulin 1 type III in peripheral nerves generate thicker myelin sheaths (Michailov *et al.*, 2004; Taveggia *et al.*, 2005). Further studies were carried out in the CNS to investigate the role of neuregulin 1 type III. *In vitro*, the addition of exogenous neuregulin 1 to myelinating co-cultures of oligodendrocytes and dorsal root ganglion (DRG) neurons showed an increased in myelination (Wang *et al.*, 2007), and myelination is significantly reduced in co-cultures of neuregulin 1 type III-deficient DRG neurons (Taveggia *et al.*, 2008). *In vivo*, transgenic mice expressing a dominant-negative ErbB receptor in oligodendrocytes and mice haplosuffficient for neuregulin 1 type III showed hypomyelination (Roy *et al.*, 2007; Taveggia *et al.*, 2008).

In the PNS, myelinating Schwann cells establish contact with a bundle of axons (a process termed radial sorting) and interdigitate processes to establish contact with unmyelinated axons in order to ensheathe an internode (Jessen and Mirsky, 2005). This process requires the intervention of β1 integrin and focal adhesion kinase (FAK, a downstream signalling molecule from the integrin pathway) (Grove *et al.*, 2007; Nodari *et al.*, 2007). In the CNS, myelination is delayed and oligodendrocytes acquire a higher threshold for myelination in dominant negative β1 integrin mice, suggesting that integrins are necessary for detection of the axonal signal that initiates myelination (Câmara *et al.*, 2009). Integrins are a major family of extracellular matrix (ECM) receptors comprising two subunits (α and β) that heterodimerize in 24 known combinations. Integrins bind ECM ligands and signal through the PI3K and MAPK pathways (Hynes, 2002) and interact with different receptors, including the ErbB receptor family that regulates neuregulin signalling, thereby amplifying neuregulin signalling.

Although there are similarities between PNS and CNS myelination, previous research has demonstrated that these two systems diverge in terms of signalling pathways that regulate myelination. CNS seems to be a more complex system which, unlike the PNS, does not have an identified factor that has an exclusively instructive role, but rather is governed by sequential events dependent on temporal expression of positive and inhibitory factors (Fig. 1.2).

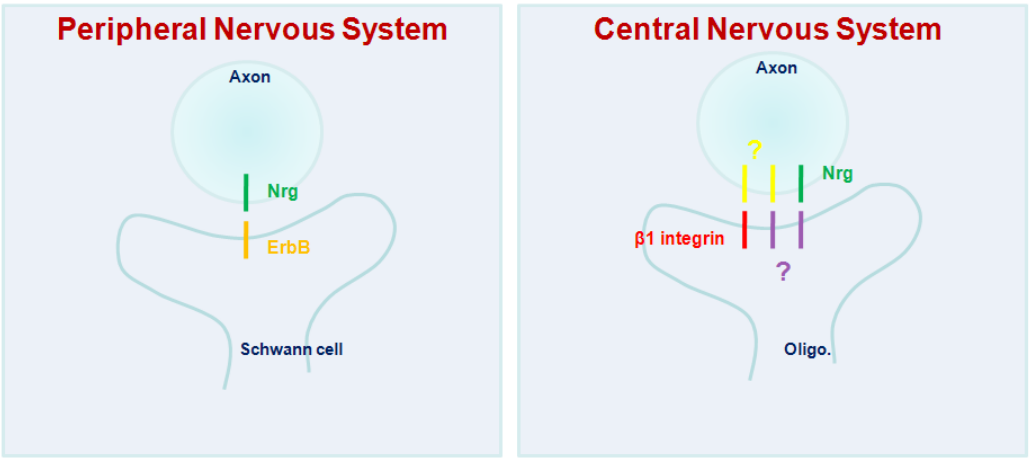


Figure 1.2– Signalling pathways regulating myelination in peripheral and central nervous system. In the peripheral nervous system neuregulin 1 type III has been identified as a key regulator of myelination, capable of controlling myelin thickness and the Schwann cell decision to myelinate. In the central nervous system such key molecule has not been described yet.

[For a more complete description of integrins see Chapter 3, and for neuregulins, Chapter 4.]

1.2.2 Composition of myelin

Myelin is highly conserved in mammals (Kirschner *et al.*, 1989; Waehneldt, 1990). Myelin is a low hydrated structure containing 40% water and its dry weight is characterized by a high proportion of lipids (70%) and a lower proportion of proteins (30%) (Baumann *et al.*, 2001; Siegel *et al.*, 1999). It has a high lipidic content with

approximately 32% glycolipids (from which 20% are of galactocerebroside, GalC), 26% cholesterol, and 42% phospholipids (Pfeiffer *et al.*, 1993).

GalC concentration in the brain is directly proportional to the amount of myelin (Siegel *et al.*, 1999). UDT-galactose ceramide galactosyltransferase (CGT) is necessary for the activation of GalC and its derivative sulphatide. In CGT knockout mice myelin formation is apparently normal, apart from some minor differences in structure and changes in axon conduction velocity with mice showing tremors and mild ataxia and developing progressive hind limb paralysis with age (Coetzee *et al.*, 1996; Siegel *et al.*, 1999). This suggests that the lack of GalC (which plays a role in the establishment of the axoglia contact) may be compensated by glucocerebroside (GlcC), explaining the absence of a severe phenotype, therefore we can conclude that it is not necessary for myelin formation but is crucial for insulating capacity and stability (Dupree *et al.*, 1998; Siegel *et al.*, 1999).

Myelin membrane contains a high percentage of lipids from which cholesterol is a major component. Cholesterol concentration in the brain abruptly increases after birth in mice due to myelin production by oligodendrocytes, going from 3.5 mg/g in the newborn to 12 mg/g after only 3 weeks and reaching its peak (19mg/g) during adulthood (Dietschy and Turley, 2004). Transgenic mice lacking the ability to synthesise cholesterol in myelinating oligodendrocytes developed ataxia, tremor and had impaired control of hind limbs and several unmyelinated axons around P20. This phenotype is transient, with most axons being myelinated by P100, improving the trembling phenotype. This transient effect is thought to be due to oligodendrocytes taking up cholesterol from astrocytes, as a compensatory mechanism for the lack of cholesterol synthesis (Saher *et al.*, 2005).

Myelin proteins

Myelin proteins account for approximately 30% of its dry weight, making myelin a biological membrane with a simple protein composition since most biological membranes have higher levels of proteins than lipids (Quarles *et al.*, 2006).

Proteolipid protein (PLP) is also known as Folch-Lees protein (Lees *et al.*, 1984). PLP is a tretaspan protein corresponding to 50% of CNS myelin proteins (Baumann *et al.*, 2001), and has the physical property of solubility in organic solvents (Siegel *et al.*, 1999). PLP is the common isoform, but the splicing of the 5' part of exon 3 (from 7 exons that constitute the PLP gene, located on the X chromosome both in mice and humans), leads to a 35 amino acid deletion that originates the DM-20 isoform (Baumann *et al.*, 2001). A variety of naturally occurring mutations affecting both PLP and DM-20 has been described in rodents, among them are *jimpy*, *jimpy* ^{msd} and *jimpy 4j* (Baumann *et al.*, 2001; Billings-Gagliardi *et al.*, 1995; Siegel *et al.*, 1999). *Jimpy* mice revealed a myelin deficiency with a reduced number and thickness of myelinated axons, with the few myelinated axons showing an abnormal compaction and no intraperiodic line (Duncan *et al.*, 1989); *jimpy* mice die prematurely (Nave *et al.*, 1986). Although one would expect PLP and DM-20 to be necessary for compact multilamellar myelin, it is now known that, although they serve important functions, they are not essential (Siegel *et al.*, 1999). PLP/DM-20 knockout mice showed a relatively normal myelin formation (Klugmann *et al.*, 1997). Oligodendrocytes were able to myelinate axons and compact myelin sheaths, despite a difference at the level of intraperiodic line, which was abnormally condensed (Baumann *et al.*, 2001; Siegel *et al.*, 1999).

Myelin basic protein (MBP) is an essential protein for myelin formation. It is a cytoplasmic, extrinsic membrane protein that constitutes 30-40% of total myelin content in the CNS and 10-20% in the PNS (Jacobs, 2005; Pfeiffer *et al.*, 1993). MBP has an important role in myelin compaction and mutations in the MBP gene cause severe dysmyelination (Pfeiffer *et al.*, 1993). MBP is encoded by a gene containing 7 exons, located on chromosome 18 both in mouse and human, from which different protein isoforms are generated by alternative splicing of RNA (Ferra *et al.*, 1985; Roach *et al.*, 1985; Siegel *et al.*, 1999). The mutant *shiverer* mouse results from one of the major deletions of the MBP gene, presenting a phenotype where hypomyelinated axons possess uncompacted myelin sheaths, with no major dense line (Siegel *et al.*, 1999). *Shiverer* mice display tremors from P10 to P12,

followed by tonic seizures, dying 4 or 5 months after birth (Baumann *et al.*, 2001; Privat *et al.*, 1979; Rosenbluth, 1980). Although these mice lack MBP in both PNS and CNS, there is no phenotype in the PNS suggesting that other proteins, such as P0, can compensate for the loss of MBP (Baumann *et al.*, 2001; Boggs, 2006).

2':3'-Cyclic nucleotide-3'-phosphodiesterase (CNP) exists in two isoforms (46 and 50kDa), representing 4% of the total of CNS myelin proteins (Pfeiffer *et al.*, 1993). CNP is highly expressed in the CNS myelin and oligodendrocytes but has a lower level of expression in Schwann cells at the initial stage of myelination. CNP is not a major component of compact myelin but is concentrated in the cytoplasm of uncompacted myelin, inner and outer tongue processes and lateral loops (Siegel *et al.*, 1999; Trapp *et al.*, 1988). Cnp1 (CNP gene that comprises 4 exons) knockouts display largely normal myelin; the mutation perturbs myelin formation and causes abnormal oligodendrocyte membrane expansion, with mice developing neurological symptoms like ataxia, hind limb weakness and hunched posture at 4 months, and dying prematurely (Baumann *et al.*, 2001; Lappe-Siefke *et al.*, 2003). Oligodendrocytes in mutant mice over-expressing human CNP extend an increased number of longer processes, although with low myelin compaction, redundant myelin and intra-myelin vacuoles (Gravel *et al.*, 1996). It has been proposed that CNP is involved in the cytoskeletal network of myelin since it binds to F-actin and tubulin, and overexpression of CNP in non-neuronal cells stimulates process outgrowth (Quarles *et al.*, 2006; Siegel *et al.*, 1999).

Myelin-associated glycoprotein (MAG) is a heavily glycosylated protein present in both PNS and CNS and is a minor constituent of myelin (approximately 1%). MAG comprises 2 isoforms generated by alternative splicing, S-MAG (with a short cytoplasmic domain) and L-MAG (with a long cytoplasmic domain) (Pfeiffer *et al.*, 1993; Siegel *et al.*, 1999). MAG has a role in initial axon-myelin interactions, since it is not present in compact myelin but is located at the periaxonal membrane. This emphasizes the role of MAG as a recognition molecule, binding ligands and mediating axoglial interaction (Pfeiffer *et al.*, 1993). MAG knockout mice display largely normal myelination, but exhibit subtle periaxonal structural abnormalities (Li

et al., 1994; Montag *et al.*, 1994; Siegel *et al.*, 1999). At 9 months, null mutants develop a dying-back oligodendropathy, a degeneration of the distal periaxonal oligodendrocyte processes, and a peripheral neuropathy affecting both myelin and axons (Lassmann *et al.*, 1997; Siegel *et al.*, 1999). A transgenic mouse, expressing a truncated form of the L-MAG, yet maintaining S-MAG expression, showed a similar CNS phenotype to the MAG KO mice, suggesting that L-MAG is essential for CNS myelination (Baumann *et al.*, 2001; Miescher *et al.*, 1997).

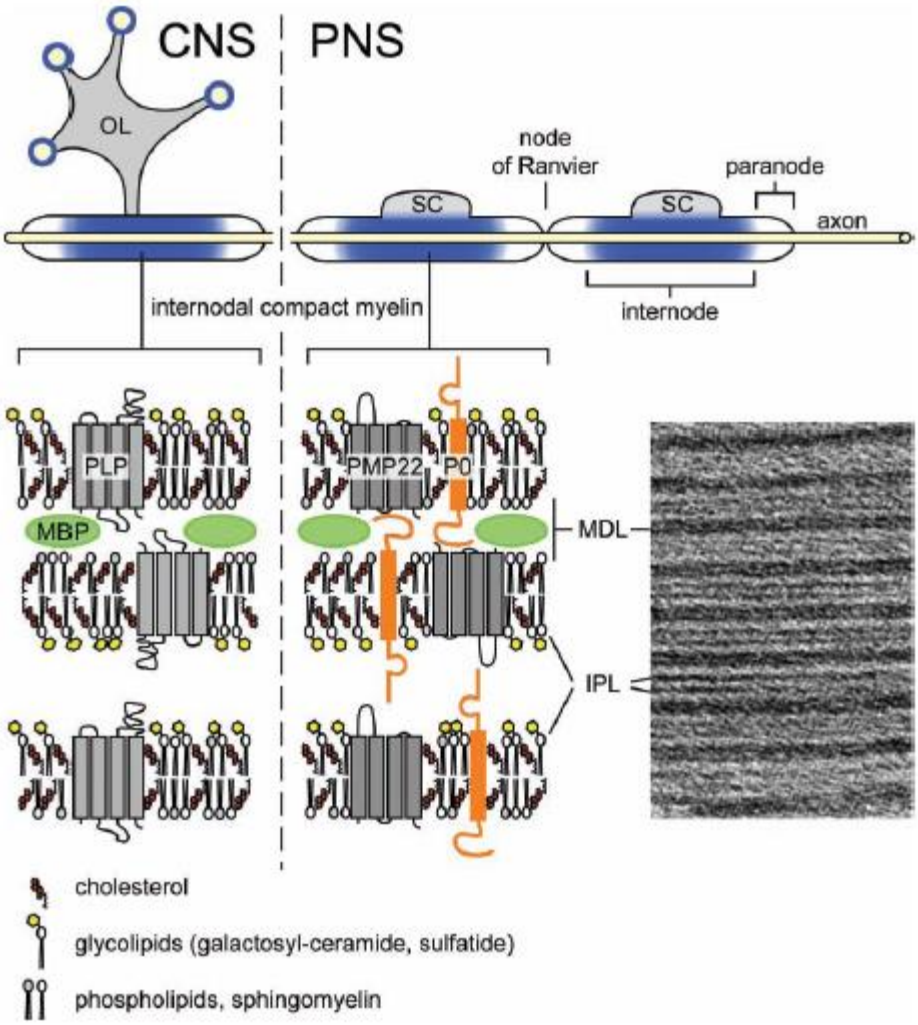


Figure 1.3 - Schematic representation of myelin composition in the central nervous system and peripheral nervous system. The myelinating cells of the central (oligodendrocyte) and peripheral (Schwann cell) nervous system differ from one another, not only in the number of internodes each one can myelinate at a time, but also the proteins synthesized. Central myelin is mainly constituted by MBP, in contrast to peripheral myelin which has P0 as its main constituent. Whilst in the PLP is present in the CNS, this protein is substituted in the PNS by PMP-22. Reproduced from Saher *et al.*, 2011

As depicted in figure 1.3, peripheral and central myelin share most of their constituents, but in the PNS, PLP function is substituted by P0 and PMP-22. Myelin protein P0 is a major constituent of peripheral myelin (Saher *et al.*, 2011). Disruption of the MPZ gene (that encodes both P0 and PMP-22) results in demyelination of peripheral nerves and will be described in the next section.

1.2.3 Myelin disorders

There are different pathologies that affect myelin which can be caused by genetic or toxicological factors, autoimmunity, viral infections or mechanical trauma. These can affect the myelin layer, the neurons or the myelinating cell itself (Quarles *et al.*, 2006). Myelin deficiencies can be divided into acquired and genetic disorders of myelin, and also into demyelinating and dysmyelinating diseases (Siegel *et al.*, 1999).

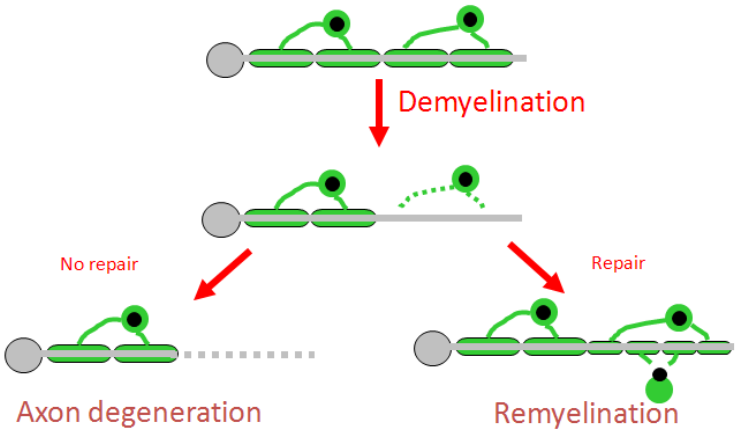


Figure 1.4 - Schematic representation of the demyelinating process. Myelin wrapped around axons is degraded due to genetic, autoimmune or toxic factors leading to the exposure of axons that without the structural support of oligodendrocytes degenerate in a situation of no repair, or in the case where a regenerative process occurs can be remyelinated. Remyelinating fibres have a thinner myelin sheath. Reproduced from Charles ffrench-Constant.

Demyelinating diseases

A *demyelinating* disease is characterized by damage and loss of the myelin sheath leading to axon degeneration. Demyelination can be caused by genetic or inflammatory factors that lead to myelin degradation which impairs the conduction of the nervous impulse that can cause movement, cognitive, visual, or sensory damage. If there is no repair these will lead to axon degeneration, although in some situations regeneration takes place and these axons are remyelinated (Fig. 1.4).

Multiple sclerosis (MS) and leukodystrophies are examples of demyelinating diseases of the CNS, while Charcot-Marie-Tooth disease and Guillain-Barré syndrome are characteristic of the PNS.

Charcot-Marie-Tooth disease (CMT) is the most frequent human inherited neuropathy of the PNS (prevalence 1:2500) caused by abnormalities in the PMP-22 gene (a glycoprotein that play a role in myelin structure and compaction). The phenotype of these mutations can lead to either increased or decreased expression of PMP-22 with similar symptoms showing the importance of tight regulation in myelin proteins (Rosso *et al.*, 2012). CMT is characterized by demyelination that slows the conduction velocity, leading to weakness and numbness (Kedlaya, 2007; Siegel *et al.*, 1999). The Trembler-J mouse has a mutation on the PMP-22 gene that confers a model for CMT due to an abnormal folding of the protein preventing normal myelination (Meekins *et al*., 2004).

Guillain-Barré syndrome (GBS) is an acquired disorder of the PNS characterized by an acute inflammatory demyelinating polyneuropathy which appears one or two weeks after a bacterial or viral infection and it is initially characterized by leg weakness and can cause paralysis (Quarles *et al.*, 2006; Siegel *et al.*, 1999). GBS is the most common cause of acute flaccid paralysis thought to be induced by autoimmunity (reviewed by Yuki and Hartung, 2012). One of the animal models used to help understand this syndrome is the experimental allergic neuritis (EAN), induced through immunization with PNS specific proteins (reviewed by Soliven,

2012). This mouse model showed that the P2 myelin protein plays an important role in the demyelinating process, although this protein has not been consistently shown to have a role in GBS (Spies *et al.,* 1995). A new line of research has been following a different route by using animal models immunized against gangliosides, which have been documented to be associated with the human GBS by reacting with a carbohydrate epitope of MAG, P0 and PMP-22 (Zhu *et al.,* 1994). Further investigation into antibodies and pathological causes linked to this disease are required for the development of better models (Quarles *et al.,* 2006).

Leukodystrophies are genetic diseases of the CNS caused by multiple genetic defects affecting specific myelin proteins and are characterized by a progressive degeneration of the white matter (Siegel *et al.,* 1999). Typically leukodystrophies have an early onset and new lines of treatment that include cell transplantation have been used, but have not been proven to promote myelination regeneration (Orchard and Tolar, 2010). Krabbe disease is a demyelinating disorder caused by a mutation of the galactocerebrosidase (GalC) gene, which prevents its degradation by oligodendrocytes and Schwann cells leading to progressive demyelination in the CNS and PNS. The animal model used in this specific type of leukodystrophy is the *twitcher* mouse that has a mutation in the GalC gene leading to a lack of GalC enzyme. This model is used to test different therapies such as oligodendrocyte precursor cells and bone marrow transplantation (reviewed by Duncan *et al.,* 2011).

Multiple Sclerosis (MS) is one of the most common inflammatory diseases of the CNS affecting 2.5 million people worldwide (Compston *et al.,* 2002). MS is characterized by inflammation, demyelination, degeneration of oligodendrocytes (that then fail to remyelinate in chronic phases of disease), resulting in progressive loss of vision and paralysis (Baumann, 2001; Siegel *et al.,* 1999). MS can be subdivided into 4 types. *Relapsing-remitting MS*, the most common subtype, is characterized by acute attacks (relapses) followed by a recovery process (remission). This subtype can then evolve to *secondary progressive MS*, which is progressive due to the absence of recovery periods. *Primary progressive MS* is less common but clinically more aggressive and defined by a steady increase of disability with no

recovery periods. The rare *progressive-relapsing MS* starts as progressive and gets worse due to super-imposed attacks (reviewed by Goldenberg, 2012). Animals immunized against myelin encephalitogenic proteins develop an acute or chronic demyelinating disease of the CNS termed experimental allergic encephalomyelitis (EAE) and are currently used as a model for MS. The different types of antigen used to immunize these animals lead to distinct models that mimic different pathological aspects or a specific subtype of MS (reviewed by Constantinescu *et al.*, 2011).

Chang and co-workers reported that human MS lesions contain oligodendrocytes that extend multiple processes that associate with demyelinated axons but fail to myelinate them (Fig. 1.5). Identification of the signals regulating the initiation of myelination may lead to the development of strategies to promote effective remyelination by oligodendrocytes within MS lesions that are arrested at the premyelinating stages and therefore unable to contribute to repair, and prevent the axon degeneration that is thought to underlie chronic disease (Chang *et al.*, 2002).

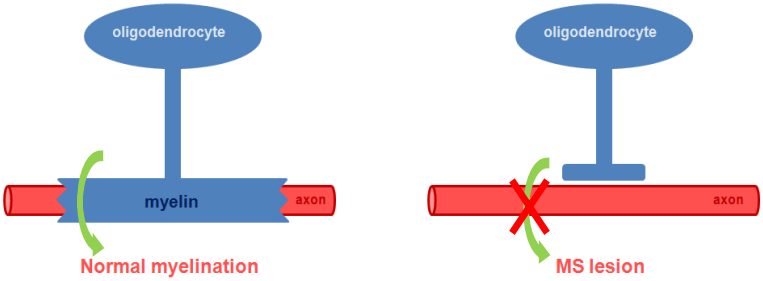


Figure 1.5 – Schematic representation of multiple sclerosis (MS) lesion. In normal circumstances the oligodendrocyte extends a process that contacts the axon and myelinates the internode. In MS lesions oligodendrocytes migrate into the lesion and establish an initial axoglial contact but fail to remyelinate.

Dysmyelinating diseases

Dysmyelination is associated with defective myelin structure and function, which is abnormal and can lead to axonal degeneration (Quarles *et al.*, 2006). Unlike

demyelinating diseases that can have different triggers, dysmyelinating disorders have a genetic background that prevents the formation of normal myelin usually due to biochemical abnormalities.

Pelizaeus-Merzbacher disease (PMD) is a rare disorder of the CNS caused by mutations in the PLP gene that encodes two proteins in the oligodendrocyte: PLP and DM20 (smaller isoform of the PLP protein). The severity of the symptoms varies depending on the mutation, with mutations that only affect PLP being less severe (Pearlman and Mar, 2012). The main symptoms of PMD are delays in coordination, motor abilities and intellectual function, although motor skills are more affected then intellectual skills, such as memory and language. Since PMD can be caused by multiple types of mutations, there are also different animal models described in the literature that attempt to mimic this pathology (Gruenenfelder *et al.*, 2011). The myelin deficient (md) rat overexpresses PLP and simulates the more severe case of PMD with demyelination and early death (Cziza and Lahunta, 1979; Jackson and Duncan, 1988). A mouse model termed *jimpy* also mimics PMD by decreased expression of PLP, with these mice showing hypomyelination and early oligodendrocyte death (Wight *et al.*, 2007). The fact that over- and under-expression of the PLP gene results in the same hypomyelinating phenotype indicates that the PLP gene is dosage sensitive (Griffiths *et al.*, 1995).

1.3 Anatomy of myelination

Many models of actual myelin wrapping and formation have been described over the years with the help of electron microscopic (EM) data. One of these models, "carpet crawler" (Sobottka *et al.*, 2011), describes the myelination process as an analogy to rolling up a carpet, where the glial cell establishes contact with the axon, defines the size of the internode, extends a process along the axon with the specific width required and rolls several layers of myelin conferring a smooth aspect to myelin (Bunge *et al.*, 1989). This model has shortcomings since other EM studies have

reported that the myelin surface is not smooth or concentrical (Knobler *et al.,* 1976). A different model that tried to reconcile these differences, termed the "serpent" model (Sobottka *et al.,* 2011), describes that myelination is made in a spiral fashion (since myelin proteins have been observed forming a helical pattern along the internode) (Brockschnieder *et al.,* 2006), wrapping myelin in a unidirectional way along the axon (Pedraza *et al.,* 2009). The divergences in both these models have recently been addressed by two different groups that suggest complementary myelination models. The fact that there are different observations is an indicator that all of these models may all be part of the myelination process.

Sobottka and co-workers published their findings describing a new myelination model termed the "liquid croissant" model based on data from high-resolution confocal time lapse imaging of organotypic cerebellar slice cultures. Myelination is described as being driven bidirectionally in a coiled manner, with the non-smooth myelin surface wrapped around the axon in a triangular shape (Sobottka *et al.*, 2011). Interestingly, another model has been described recently based on time lapse analysis of *in vitro* (myelinating cultures of mouse spinal cord) and *ex vivo* (spinal cord explants of *shiverer* mice) data. In this model myelination is defined by wrapping in a corkscrew fashion followed by a longitudinal extension of the membrane to form the concentrical structure observed by EM (Ioannidou *et al.,* 2012).

The fact that all of these models depict a different way of wrapping and extending processes suggests that the entire process of myelin formation is a very complex one that might encapsulate either different forms of myelination in different areas of the brain, or that all of these models are complementary and represent different stages of the myelination process.

Oligodendrocytes and Schwann cells have the ability to myelinate different sizes of axons. While the Schwann cell myelinates only one internode it is known that these can have different lengths; the same applies to oligodendrocytes that extend and contact multiple axons and myelinate different internodes at one time. The complexity of this problem has been a long-lasting debate, trying to understand if

this is an intrinsic ability of the glial cells, if it is controlled by the axon, or if this process is influenced mutually by glial cells and neurons.

Friede showed that there is a tight relation between the diameter of the axon and the thickness of the myelin sheath, termed the g-ratio, characterized by the ratio between the inner and outer circumferences of the myelin sheath (Fig. 1.6) (Friede, 1972). The g-ratio is an indicator of optimal axonal myelination with its value representing the optimal myelin thickness required to achieve maximum efficiency of conduction. The optimal g-ratio value can be theoretically calculated by taking into account axonal volume. In 2009, it was reported that the optimal g-ratio value for CNS is approximately 0.77, whilst in the PNS is approximately 0.6 (Chomiak and Hu, 2009).

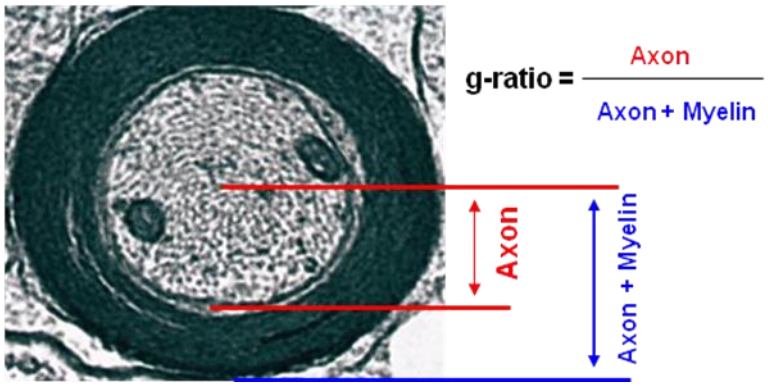


Figure 1.6 – G-ratio as an indicator of optimal axonal myelination. The ratio between the axon diameter and the axon diameter with the myelin layer (g-ratio) is used to analyse myelin morphology and thickness.

With a different range of axon diameters (although the diameter is maintained along the length of the axon (Friede and Martinez, 1970)), the question remains: how does a glial cell determine which axon to myelinate and how many wraps to make?

One of the approaches taken by Elder and co-workers was to generate mutant mice lacking either midsize or heavy neurofilament subunits, or both, leading in all three cases to a decrease in axon diameter of myelinating axons. This work showed that the intrinsic process of establishing myelin sheath thickness differs from oligodendrocyte to Schwann cells. In these mice, the PNS fibres were hypermyelinated: the amount of myelin wrapped around the axons corresponded to

the thickness observed in normal caliber axons. These shows that Schwann cell-mediated myelination is not dependent on axon size. As for the CNS, oligodendrocytes are more complex due to their ability to establish multiple internodes on different axons that might have different diameters, thus demanding a more complex mechanism where each process has to contact an axon and decide the correct amount of myelin to generate. Interestingly, these mice do not show major myelinating defects in the CNS, with oligodendrocytes myelinating each axon with the correct number of sheaths expected for its diameter, which demonstrates the oligodendrocytes' ability to adjust its myelin production to axonal size (Elder *et al.*, 2001).

Recent research has proposed a mechanism by which oligodendrocytes regulate myelin sheath thickness. Furusho and co-workers analyzed two lines of mice that lacked the fibroblast growth factor (FGF) receptor 1 and receptor 2 in oligodendrocytes. FGF has previously been shown to have a role in oligodendrocyte precursor cell proliferation and differentiation (reviewed by Webster, 1997). These mice show no defect in terms of proliferation or differentiation, with the main phenotype being hypomyelination and decreased expression of major myelin genes, such as MBP and PLP. Due to the fact that no changes where seen in the earlier stages of myelination, this work suggests that FGF receptors are important regulators of late stages of myelination by regulating the amount of sheaths wrapped around axons in accordance to diameter (Furusho *et al.*, 2012).

Oligodendrocytes arise from distinct regions of the subventricular zone and have different migratory pathways, with some oligodendrocytes making their way to the optic nerve (area characterized by small diameter axons) or spinal cord (area that comprises a mixture of small and large axons) for example. It was thought that oligodendrocytes had an intrinsic commitment to myelinate the type of axon caliber related to the region from which they originated. In 1998, Fanarraga and co-workers reported that oligodendrocytes are not committed to myelinate specific sizes of axons, using transplantation of oligodendrocytes from the optic nerve into demyelinating regions of the spinal cord. If these oligodendrocytes had the innate

ability to only myelinate a specific size of axon and wrap a specific number of sheaths, they would retain this property and only myelinate the smaller axons in the region, or only produce a set amount of myelin sheaths around any given axon. This experiment showed that oligodendrocytes are actually able to adapt and judge the axonal dimensions of the neurons they contact, since the oligodendrocytes from the optic nerve could correctly identify the size of the axon and myelinate accordingly (Fanarraga *et al.*, 1998).

Other animal models of myelination can also prove very useful in answering this question. Zebrafish has been established as one of these models and recent work where mutated fish acquire several extra Mauthner axons (zebrafish possess two of this large caliber axons, one for each half of the body) showed that oligodendrocyte myelination can be regulated by an increased number of axons. Oligodendrocytes that normally would only myelinate one of these axons were able to myelinate several of this very large caliber axons. Additionally, oligodendrocytes that would commonly myelinate only small diameter axons could also myelinate large caliber ones. This represents a new perspective on the idea that oligodendrocytes could only myelinate either several small caliber axons, or few large diameter axons, since this work shows that oligodendrocytes have the ability to myelinate different sizes of axons simultaneously (Almeida *et al.*, 2011).

1.4 Molecular regulation of myelination

Myelination is characterized by the extension of processes, selection of the axons to myelinate, initiation of *axoglial contact*, followed by the initial *wrapping*, regulation of myelin thickness and assembly of the nodes of Ranvier, and finally the *compaction* of myelin (Fig. 1.7).

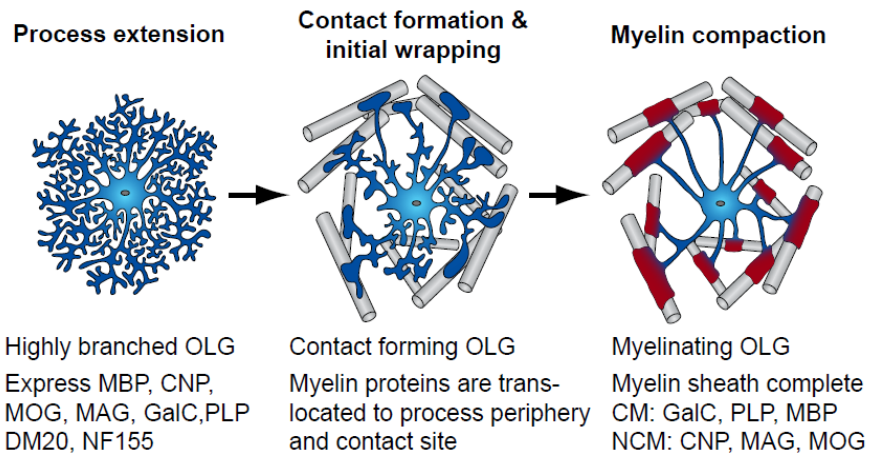


Figure 1.7 – Different stages of oligodendrocyte development. Oligodendrocytes extend several processes and contact axons in order to initiate myelination. Oligodendrocytes are able to sense the axon diameter and ensheath the correct number of wraps and form the compact myelin sheath.
Reproduced from http://www.crm.ed.ac.uk/research/group/myelination-and-repair-cns

1.4.1 Initiation – axoglial contact and extension

One of the most crucial events in myelination is axoglial contact. Oligodendrocyte development comprises a series of overlapping stages during which the cells migrate and proliferate. Oligodendrocytes able to establish axonal contact will differentiate and become myelinating cells, while those who fail undergo apoptosis (Barres *et al.*, 1992, Trapp *et al.*, 1997). Cells which eventually become myelinating cells associate with an axon, establish axoglial contact, define the appropriate myelin thickness, and extend membranes in order to wrap the myelin sheath.

Throughout the nervous system it is possible to encounter myelinated and unmyelinated axons. The reason why some axons are kept unmyelinated is not fully understood, although it is known that there is a diameter threshold for axons to be myelinated (1 µm in the PNS and 0.1 µm in the CNS) (Waxman and Bennett, 1972; Voyvodic, 1989). This suggests that the axon controls the signal read by the glial cell which needs to assess whether or not that axon is meant to be myelinated. It has been

proposed that these small caliber axons are not myelinated in accordance to physiological properties, since being ensheathed by a myelin layer probably would not enhance the rates of nerve impulse conduction (reviewed by Sherman and Brophy, 2005). These unmyelinated fibres express *L1* and neural cell adhesion molecule (NCAM) which are downregulated in myelinated axons, which would be an indicator that these adhesion molecules negatively regulate myelination (Charles *et al.,* 2002; Haney *et al.,* 1999). It is known that L1 can regulate axonal outgrowth and maintenance of axoglial interactions in both PNS and CNS (Nave and Trapp, 2008; Coman *et al.,* 2005) but since the L1 knockout mouse shows no defects in myelination (Cohen *et al.,* 1998), it is currently not possible to conclude whether L1 is responsible for determining whether an axon will be myelinated.

Unlike the PNS, where growth factor *neuregulin 1 type III* has been identified as a pivotal signaling molecule that regulates myelination (Michailov *et al.,* 2004; Taveggia *at al.,* 2005), in the CNS such a signal remains to be identified. Neuregulins and their ErbB receptor family are key regulators of Schwann cell development and myelination (reviewed by Birchmeier and Nave, 2008; Birchmeier, 2009). Neuregulin 1 type III is expressed on the axonal surface and in the PNS can regulate whether an axon is myelinated and the myelin thickness (Michailov *et al.,* 2004; Taveggia *at al.,* 2005). Studies in the CNS on neuregulin 1 type III are controversial and do not seem to support this same role as in the PNS. A full description of neuregulin regulation and the PNS/CNS paradigm will be further discussed in Chapter 4.

Integrins (and their ligand: laminins) are also candidates to play a role in the initiation of myelination. Laminin-2 can enhance myelination in oligodendrocytes in culture (Buttery and ffrench-Constant, 1999) and the lack of lamininα2 in mice and humans leads to a dysmyelinating phenotype (Jones *et al.,* 2001; Chun *et al.,* 2003). It has been established that $\alpha 6\beta 1$ integrin, a receptor for laminin expressed by oligodendrocytes at the time of myelination, regulates oligodendrocyte survival signaling by amplification of neuregulin activity (Colognato *et al.,* 2002). Furthermore, integrin $\beta 1$ can delay initiation of myelination on small caliber axons.

Using a dominant negative β1 integrin under control of the MBP promoter (expressed only in oligodendrocytes), it was observed a reduction in the number of internodes produced by each oligodendrocyte suggesting that these oligodendrocytes produce less myelin, and a higher axonal diameter threshold is required for myelination (Câmara *et al.,* 2009). An overview of the role of integrins and associated signaling pathways will be presented in Chapter 3.

The activity of GTPases such as Cdc42, Rac1 and RhoA (expressed by oligodendrocytes (Esrchbamer *et al.,* 2005)) can be modulated by integrins which influence oligodendrocyte development. Liang and co-workers (2004) showed that Cdc42 and Rac1 can promote process extension and branching, on the other hand RhoA seems to have the opposite effect and act as an inhibitor (Liang *et al.*, 2004). Two years later it was described that Cdc42 and Rac1 have a stage-specific and crucial role in myelination. When Cdc42, Rac1, or both, where inactivated in CNP-expressing oligodendrocytes, myelination was abnormal, presenting outfoldings due to a defect in compaction with accumulation of cytoplasm that progressively worsened with age (Thurnherr *et al,.* 2006). Since both these GTPases are known cytoskeletal regulators, their ablation during myelination and the consequent phenotype shows that cytoskeletal organization is necessary for cytoplasm exclusion and myelin compaction.

Neurotrophins, a family of target-derived growth factors, also play a role in the initial stage of myelination. The brain-derived neurotrophin factor (BDNF) promotes myelination by binding to glial p75 receptor (Chan *et al.,* 2006). In the PNS, BDNF binds to the truncated tropomyosin receptor kinase (Trk) B to limit myelination whilst in the CNS, BDNF binds to the full-length Trk molecules and promotes differentiation (Xiao *et al.,* 2009). Nerve growth factor (NGF), a prototypical neurotrophin has contrasting effects in Schwann cells and oligodendrocytes. NGF promotes myelination in the PNS and inhibits it in the CNS, which indicates that these cells respond differently to the same axonal signals (Chan *et al.,* 2004).

Netrins are a family of secreted molecules. Netrin1 is widely expressed by neurons and oligodendrocytes in the CNS of adult mammals (Ellezam *et al.,* 2001; Manitt *et al.,* 2001). Netrin1 and its receptor deleted in colorectal cancer (DCC) have been described as important factors for axonal path finding, acting either as attracting cues (Kennedy *et al.,* 1994; Metin *et al.,* 1997) or as repellents (Colamarino and Tessier-Lavigne, 1995; Guthrie and Pini, 1995). Other than affecting glial recruitment, netrins have also been shown to a have a role in CNS myelination by promoting oligodendrocyte branching and membrane extension via Fyn and RhoA (Rajasekharan *et al.,* 2009).

There is a current debate as to whether electrical activity of axons is required to initiate myelination. Axonal *electrical activity* has been shown to modulate oligodendrocyte proliferation and myelination. Adding tetrodotoxin (TTX), which blocks sodium-dependent action potentials, decreases OPC numbers by 80% (Barres and Raff, 1993) and blocks myelination *in vivo* and *in vitro* (Demerens *et al.,* 1996; Stevens *et al.,* 2002). A recent study where DRG neurons were treated with botulinum toxin A (BnTX) showed activity-dependent regulation of MBP synthesis which regulates initiation of myelin formation by oligodendrocytes on electrically active axons (Wake *et al.,* 2011). However, oligodendrocytes are able to myelinate fixed neurons which lack axonal activity (Rosenberg *et al.,* 2008), suggesting that other axonal factors may encourage myelination. In the PNS, release of adenoside triphosphate (ATP) dependent on axonal activity acts on purinergic receptors and inhibits myelination (Stevens and Fields, 2000).

Myelination requires a fine balance between positive and inhibitory signals to successfully control the timing of myelination. One of these inhibitory factors is *Notch*, a cell surface protein family that comprises four members. Its signalling pathway is crucial for glial cell development and myelination in the nervous system. The type of ligand defines whether the signalling pathway will follow the canonical or non-canonical route, with Jagged1 being responsible for the canonical and Deltex for the non-canonical signalling pathway. Oligodendrocytes only express Notch1, known to inhibit both oligodendrocyte maturation and myelination (reviewed by

Jurynczyk and Selmaj, 2010). When OPCs were cultured with Jagged1 expressing cells or a soluble form of Jagged1, they fail to extend processes and differentiate (Wang *et al.,* 2008; John *et al.,* 2002). In mice with deficient expression of Notch1 signalling, myelination is increased (Givogry *et al.,* 2002), whilst blocking Notch1 in OPCs leads to promotion of differentiation (Zhang *et al.,* 2009). Notch influences oligodendrocyte differentiation and myelination in a time-dependent manner, since there is a decline of Jagged1 expression during development in the rat optic nerve (Wang *et al.,* 1998). In the PNS, Schwann cells express Notch1, whilst Jagged1 is expressed by Schwann cells and neurons. Notch 1 promotes Schwann cell maturation but inhibits myelination in the PNS (Woodhoo *et al.,* 2009).

1.4.2 Wrapping

In order to successfully myelinate an axon, glial cells have to synthesize large amounts of myelin proteins and lipids, trigger the polarisation of plasma membrane in order to form myelin, and contact an axon to myelinate. After the axoglial contact has been established, the glial cell has to wrap the axon with the correct amount of layers to properly insulate the neuron. Zebrafish studies showed that process extension is a dynamic process with oligodendrocytes establishing axoglial contact but also retracting some of its processes before fully committing to myelination, which can be explained by the need to regulate a constant spacing of oligodendrocytes and uniform nodes (Kirby *et al.,* 2006).

During myelination glial cells undergo a dramatic change of shape mediated by the reorganization of the actin cytoskeleton (Novak *et al.,* 2011). The oligodendrocyte cytoskeleton comprises microtubules and microfilaments, but unlike Schwann cells, do not have intermediate filaments. In the CNS, microtubules accumulate in the cell body and myelin processes and are involved in primary process outgrowth and basic process stability, whilst microfilaments extend continuously from the cell body to the cells' periphery and are responsible for their long term stability (reviewed by Bauer *et al.,* 2009).

Despite the fact that it is relatively easy to observe initial stages of myelination, the late process of wrapping is difficult to examine. Much research has been carried out regarding oligodendrocyte development, but not much is known about the wrapping and compaction processes. Brockschnieder and colleagues used microarray expression profiling of oligodendrocyte-ablated mutant mice (where oligodendrocytes express a diphetheria toxin receptor and mice were treated with the toxin) to delve into the mechanisms of wrapping and compaction. A cytoskeletal protein expressed exclusively in oligodendrocytes was identified, Ermin. This protein is mainly expressed during late stages of myelination (expressed exclusively in MBP expressing oligodendrocytes) suggesting a role in the cytoskeleton rearrangement necessary for myelin wrapping and/or compaction. Ermin binds to F-actin and reorganizes it, showing the ability to modify cell shape and induce process extension (Brockschnieder *et al.*, 2006).

Contrary to the CNS, in the PNS different signals have been shown to have a role in process extension and wrapping. When Schwann cells' cytoskeleton is disturbed via actin polymerization inhibitor cytochalasin D or by blocking myosin II, myelin wrapping decreases (Fernandez-Valle *et al.*, 1997; Wang *et al.*, 2008). Different members of the Rho-GTPase family have been implicated in the process of wrapping. Process branching and intermodal length are regulated by Rho signalling (Pereira *et al.*, 2009; Melendez-Vasquez *et al.*, 2004), while Rac1 in conjunction with β1 integrin regulate process extension (Benninger *et al.*, 2007) and Cdc42 is involved in axon ensheathment and promotes Schwann cell decision to become a myelinating cell (Benninger *et al.*, 2007; Krause *et al.*, 2008). Recent data has identified the Neural Wiskott–Aldrich syndrome protein (N-WASP) as a regulator of longitudinal extension, membrane wrapping and myelination in the PNS (Novak *et al.*, 2011).

1.4.3 Compaction

The process of myelin compaction that occurs after wrapping is characterized by the extrusion of cytoplasm towards the edges of each myelinated internode and the formation of the node of Ranvier. The compaction of the several membrane wraps is formed by the apposition of external surfaces (named intraperiod lines) and internal surfaces (termed major dense lines) of the glial cell. This periodic ultrastructure can be observed by electron microscopy, where it is possible to identify the periaxonal space (extracellular gap between the first layer of myelin and the axon) and an area of uncompacted myelin which contains cytoplasmic matrix, enriched with specific proteins and forming the paranodal loops. Glial cells wrap myelin in the internode leaving unmyelinated gaps where Na^+ channels cluster, the node of Ranvier, where action potentials are generated (Pfeiffer *et al*, 1993) (Fig. 1.8).

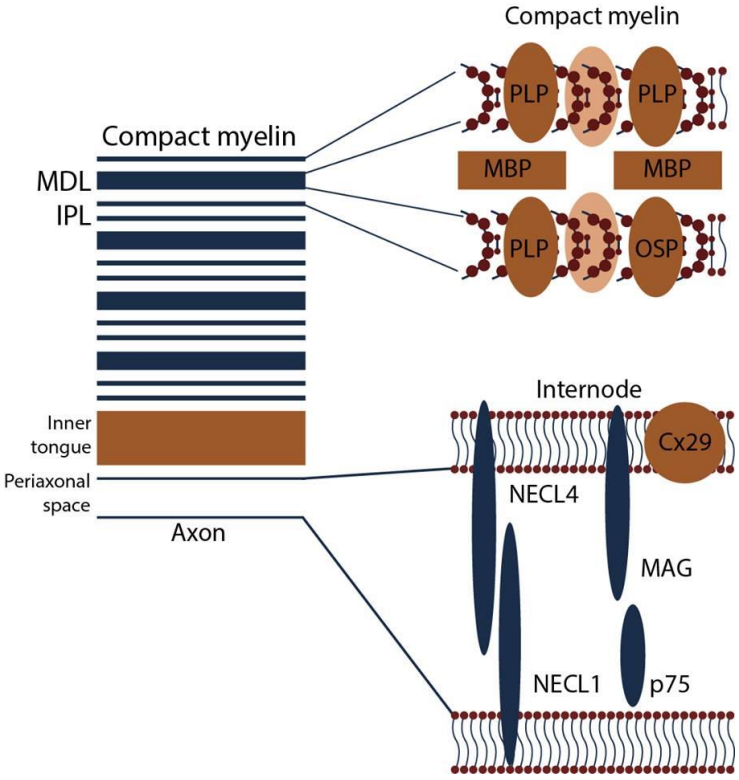


Figure 1.8 – Structural composition of compact myelin and its proteins. Compact myelin is formed by the fusion of the intermodal glial membranes, comprising the intraperiod lines (IPL – represented as a double structure) and the major dense lines (MDL). Adapted from Jahn *et al.*, 2009

2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP) is one of the markers used for myelinating oligodendrocytes. CNP is present in the cytoplasm of the myelin membrane of oligodendrocytes but it is absent in compacted myelin. It has been previously described to impact oligodendrocyte migration, membrane expansion during myelination and also to inhibit MBP accumulation and myelin compaction (reviewed by Fulton and Campagnoni, 2010). Trapp and co-workers described the localization of CNP in high concentration in the developing oligodendrocyte ahead of myelin compaction (Trapp *et al.,* 1988) and transfection of CNP into non-myelinating cells promotes the production of filopodia and process extension (De Angelis and Braun, 1994). Transgenic mice overexpressing CNP produce an abnormal myelin membrane, and compact myelin has a decrease in major dense lines (MDL) which have reduced expression of MBP and increased levels of CNP (Yin *et al.,* 1997). The observation that CNP affects myelin compaction is also consistent with the phenotype observed in shiverer mice that lack MBP, which have a complete absence of MDL (Roach *et al.,* 1983).

CNP expression can also be modulated by thyroid hormone (TH), since oligodendrocytes express active receptors for TH (Fierro-Renoy *et al.,* 1995), which regulates the synthesis of enzymes crucial for expression of myelin proteins and lipids (Rodriguez-Peña *et al.,* 1993). Neonatal hypothyroidism presents itself with delayed myelinogenesis, diminishing number of myelinated axons, and decreased myelin gene expression (Guadaño Ferraz *et al.,* 1994; Muñoz *et al.,* 1991). The myelin markers appear in a timely sequential manner, with CNP expression occurring prior to the expression of MBP, PLP (Lemke, 1993) and MOG (Pfeiffer *et al.,* 1993) in oligodendrocyte development. In the hypothyroid rodent model CNP expression is transiently impaired (Kanfer *et al.,* 1989). Myelinogenesis is dependent of TH and PDGF. In the presence of PDGF but absence of TH, OPC proliferation and differentiation are blocked. The rescue experiment where TH is added only promotes differentiation (Barres *et al.,* 1994). Further studies in hypothyroid rodent models showed a delayed expression of CNP, an abnormal distribution of CNP that resulted in less myelin compaction, and an ablation of the number of CNP expressing axons (Barradas *et al.,* 2000).

The glycoprotein P0 is the most abundant protein in PNS myelin and plays a crucial role in myelin compaction and stability. Rodent models with mutations in the MPZ gene, that encodes P0, lead to myelin disruption (Uyemura *et al.,* 1994; Warner *et al.*, 1996). Studies using an *in vitro* membrane-mimetic system showed that P0 needs to undergo a specific conformational change in order for myelin compaction to be successful and promote myelin stability. This conformational change of the P0 protein occurs faster when in the presence of cholesterol, which is abundant in the myelin layer, suggesting an interaction between proteins and lipids from the myelin membrane to achieve compaction (Luo *et al.,* 2007). Similarly to the CNP mutant mice, Martini and co-workers have described that double mutations on the P0 and MBP protein lead to absence of MDL in the PNS. However, when mice are deficient only in P0, they can partially compact myelin. MBP deficiency shows the presence of MDL in compacted myelin, suggesting that both proteins engage in the process of MDL generation (Martini *et al.*, 1995).

Evolution of myelination enabled a faster propagation of the nervous impulse with a decreased energy requirement, when compared to unmyelinated fibres. The rapid conduction allowed by myelination was crucial for the development of a complex, yet compact, nervous system but myelination and glial cells conferred other advantages such as maintenance of neuronal integrity and survival. This property is independent of myelination and is due to the lifelong interaction between the glial cell and corresponding axon. Mice with mutations in the PMP22 (*trembler* mice) and MBP (*shiverer* mice) proteins present a dysmyelinating phenotype that translates into a reduction of axonal calibre (Brady *et al.,* 1999; de Waegh *et al,* 1992), suggesting an additional role for myelination other than enabling fast conduction. *Trembler* mice show demyelination in large calibre axons that undergo repeated myelination and demyelination, resulting in little or no compact myelin explained by changes in the cytoskeleton (Low, 1976a,b). Further studies in other models of PNS hypomyelination corroborate the view that myelination regulates cytoskeleton assembly (Cole *et al.,* 1994; Hsieh *et al.,* 1994; Mata *et al.,* 1992). In the CNS, similar studies were carried out by analysing the *shiverer* mouse (which owes its name due to their "shivering" phenotype). These mice lack compact myelin in the

CNS due to the absence of MBP and show no significant alterations in the PNS apart from a mild hypomyelination (Gould *et at.,* 1995), this might be explained by compensation of loss of MBP by P0 (Rosenbluth, 1980). These mice present a decreased rate of axonal transport, altered cytoskeletal structure, decreased axon diameter and neuronal gene expression, arguing that assembly of compact myelin is necessary for maturation of neuronal cytoskeleton in large diameter axons of the CNS (Brady *et al.,* 1999). MBP homozygotic mice express approximately ¼ of normal MBP levels and have a higher g-ratio (ie, thinner myelin sheaths) but these seems to be enough to alleviate the "shivering" phenotype and increase life span (Readhead *et al.,* 1987).

Myelination comprises an initial stage of axonal recognition, where the glial cell establishes contact with the axon to myelinate. Intrinsic and extrinsic cues allow the glial cell to sense the axon calibre, extend processes and start the ensheathment of the internode, leaving unmyelinated gaps (node of Ranvier), assembling the paranodal loop and compacting myelin by extrusion of cytosol followed by the elongation of the myelin unit (Fig 1.9). Myelin not only enhances nerve conduction but also provides neuronal support. Neuronal loss is often a result of long term demyelination although observations of demyelinating models has demonstrated that demyelination is not equivalent to axonal loss. Since we cannot establish an interdependent mechanism between the degree of demyelination and axon loss, it is evident that other mechanisms other than myelin loss are involved in axon degeneration (reviewed by Nave and Trapp, 2008). Lappe-Siefke and colleagues examined transgenic mice with inactivated CNP protein and showed that this protein is essential for axonal survival. These mice demonstrated axonal swelling and neurodegeneration, but the structure of the myelin was not compromised, showing evidence that myelination *per se* is not sufficient to promote axonal survival, it is also necessary for glial support (Lappe-Siefke *et al.*, 2003).

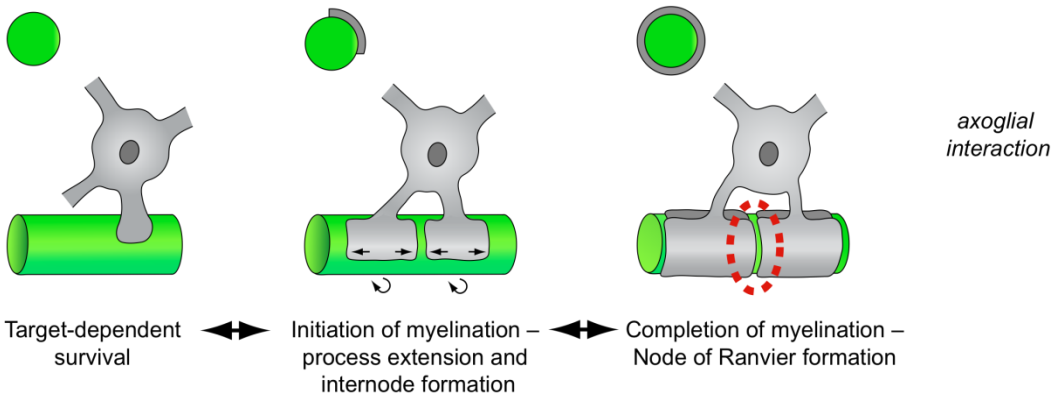


Figure 1.9 – Overview of the myelination process. Oligodendrocytes differentiate into myelinating glia and upon axonal contact extend a lipidic membrane, myelin, that provides insulation and a faster propagation of the nervous impulse between the nodes of Ranvier. Myelination is a tightly regulated process with oligodendrocytes wrapping only the necessary number of myelin sheath necessary to enable rapid impulse conduction. Image kindly provided by Dr. Nina Bauer.

1.5 Project overview

The nervous system and myelination have been thoroughly investigated over the years. It is clear that although involving different glial cells, PNS and CNS myelination have diverged from the same process and share the same function: enhancing impulse propagation. PNS has been the most described and researchers continusouly attempt to apply these observations to central myelination, but it is clear that a far more complex process occurs in oligodendrocytes, either as a preventative mechanism to avoid major impairments or due to the natural evolution of these cells. Myelin membrane is one of the most studied membranes due to the fact that it is easy to isolate and purify, which has enabled us to understand its composition, but far more remains unknown. So far, the scientific community is still trying to find an adequate model for myelination and understand how the deposition of myelin occurs.

One of the key features of myelination is the axoglial contact. Glial cells need to establish contact with an axon and wrap a precise number of myelin sheaths to

ensure an appropriate insulation of the axon. The work described in this thesis attempts to clarify some of the mechanisms involved in the axoglial contact by showing that β1 integrin regulates the initial axoglial contact and provides a threshold for myelination. In the PNS, such molecules that regulate the myelination process have been previously described. Neuregulin 1 type III can regulate the myelin thickness and the overexpression of neuregulin 1 type III in the PNS leads to myelination of axons that are normally unmyelinated (Taveggia *et al.*, 2005). In the CNS, it is thought that a complex of molecules and parallel pathways regulate the initiation of myelination. Our hypothesis is that β1 integrin would act in concert with neuregulin 1 type III, but no effect was shown, leading to the conclusion that there are compensatory mechanisms to the disruption of signalling of both molecules.

Interestingly, although neuregulin 1 does not regulate myelination in the CNS, it has been linked as a genetic susceptibility factor for schizophrenia. Analysis of post-mortem brains of schizophrenic patients shows an increased expression of neuregulin 1 type IV. Patients often show decreased white matter volume that is thought to direct a loss of cognitive skills. Here we show that overexpression of neuregulin 1 type IV in mice does not affect the integrity or density of white matter.

Central hypothesis

The central hypothesis of this PhD project is that integrins and neuregulins regulate the axoglial signals that control myelination in the central nervous system.

Statement of aims

1. To characterize myelination in the dominant negative β1 integrin mice by electron microscopy
2. To characterize myelination in transgenic mice used as control for dominant negative β1 integrin mice (ie, dominant negative β3 integrin mice, β1 integrin knockout mice, focal adhesion kinase knockout mice) by electron microscopy
3. To characterize myelination in mice crossed between dominant negative β1 integrin and neuregulin 1 type III heterozygous mice by electron microscopy

4. To characterize myelination in mice overexpressing neuregulin 1 type IV by electron microscopy and immunohistochemistry.

Chapter 2

Materials and Methods

Chapter 2

Material and Methods

This chapter describes general materials and methods used for the work this PhD thesis concerns.

2.1. Transgenic mice (dominant negative β1, dominant-negative β3, β1 integrin knockout, FAK knockout, dominant negative β1 integrin// neuregulin 1 type III$^{+/-}$, neuregulin 1 type IV overexpressing mice)

Dominant negative β1 (dnβ1) and β3 (dnβ3) mice were produced by Dr. Joana Câmara under the MBP promoter and are described elsewhere (Câmara *et al.*, 2009). Focal adhesion kinase (FAK) knockout mice were provided by Professor Peter Brophy's group, in Edinburgh, under the CNP promoter and are described by Grove and co-workers (Grove *et al.*, 2007). Promoters drive specific expression in pre-myelinating oligodendrocytes. β1 integrin knockout mice were provided by Dr. João Relvas's group, in Zurich, using the CNP promoter and have been previously described by Benninger and co-workers (Benninger *et al.*, 2006). Neuregulin 1 type III$^{+/-}$ mice were provided by Dr. Carla Taveggia in Milan, and have been described in Wolpowitz *et al.*, 2000. Neuregulin 1 type IV overexpressing mice were provided by Dr. Amanda Law (Maryland, USA) and have been described elsewhere (Deakin *et al.*, 2009). With exception for dnβ1, dnβ3 and dnβ1//neuregulin 1 type III$^{+/-}$ mice all other mice were bred, genotyped and perfused in the respective labs that provided them and tissue was shipped to our lab for dissection and processing for electron microscopy.

All animal work was done in accordance with the UK Animals (Scientific Procedures) Act, 1986 and had local ethical approval. Animals were kept in contained cages with *ad libitum* water and food supplies, and a 12h light/dark cycle.

Animals were not randomized due to the genetic nature of the experiments, since most of the mutations are either chromosome linked or have a 50% inheritance. Littermates were used as transgenic and control, since the transgenic approach was not dominant. Unless otherwise stated, all animals used in the work described in this thesis were males. All experiments were done blind, all male offspring was genotyped under codes which were only revealed in the end of the analysis.

2.2 Genotyping

2.2.1 Dominant negative β1 and β3 integrin mice

Ear notch biopsies were collected from animals and incubated in 1 mg/ml of proteinase K (Sigma) containing lysis buffer (100 mM Tris-HCl, pH 8.5, 5 mM EDTA, pH 8.0, 0.2% SDS, 200mM NaCl) overnight at 55ºC to digest the tissue and destroy proteins. The following morning, samples were vortexed vigorously and boiled at 95ºC for 10 minutes to inactivate Proteinase K. A 1 in 20 dilution of the previously described sample was prepared to be use in the PCR reaction. For each sample two different reactions were prepared, one to identify the wildtype allele (Primers: Pf - HPRT mus (5' - gagggagaaaaatgcggagtg - 3') and Pr - HPRT mus (5'-ctccggaaagcagtgaggtaag - 3')) and the other for the transgenic allele (IL2R/MBP Primers: Pf - ESIL2RB1II (5'-cagagcttgtgcattgacattg-3') and Pr: pE9.5 (5'-ggctgcaggaattcgatatca - 3')), both producing a 300 bp band.

PCR reactions were prepared to a final volume of 25 μl, according to the following ratios: 13.65 μl of PCR water, 2.5 μl of Buffer Taq, 1 μl dNTPs (stock at 10 mM), 1

µl Pf, 1 µl Pr, 1.6 µl DMSO, 4 µl DNA and 0.25 µl of Taq polymerase. A negative control reaction was prepared in parallel with the volume of DNA substituted by distilled water.

The PCR reactions were submitted to a PCR thermocycle of:

-94 °C 3 minute

-94 °C 1 minute

-58 °C 1 minute

-62 °C 1 minute

-72 °C 1 minute

* repeated the last 4 steps 29 times

-72 °C 10 minutes

The products of the PCR reaction were run in a 1.5% - agarose/TBE gel together with a 100bp DNA ladder.

2.2.2 Dominant negative β1 integrin//neuregulin 1 type III$^{+/-}$ mice

These mice had to be genotyped for both transgenes: dominant negative β1 integrin (explained above) and for the neuregulin 1 type III mutation (detailed below).

Tail pieces were collected from mice (0.6 - 0.8 cm) and digested overnight at 55°C in lysis buffer (100 mM Tris-HCl, pH 8.5, 5 mM EDTA, pH 8.0, 0.2% SDS, 200mM NaCl) with 5 ml of 10mg/ml Proteinase K. The following morning the DNA was extracted with 500 µl of phenol/chlorophorm/isoamyl alchol 25:24:1. The DNA was precipitated with ice cold 100% EtOH followed by one wash in cold 80% EtOH and finally re-suspended in 200 µl TE.

A PCR reaction was performed using the GO Tak kit (Promega) according to manufacturer instruction. Succinctly: a PCR reaction of a final volume of 25 µl was

prepared with 5 µl GoTaq Flexi Buffer 5X (Promega), 2.5 µl $MgCl_2$ (25mM), 1 µl dNTP mix (10mM), 1 µl 1960GUP (5'- actttcttcttcccattctgt -3'), 1 µl 3622GWT (5'- tttactcttcctttacggtcta -3'), 1 µl 2462GMUT (5'- tttctcttgattcccactttg -3'), 1 µl DNA solution, 12.25 µl of H_2O for PCR and 0.25 µl GoTaq Polymerase (Promega).

The PCR sample submitted to a PCR thermo cycle:

- 94ºC 4 minutes
-94ºC 30 seconds
-72ºC 1 minute
-72ºC 1 minute
* The last 3 steps repeated 35 times
-72ºC 7 minutes

The PCR product was run on a 2% agarose – TBE gel for 1 hour at 100V, with a 100bp DNA ladder.
The wildtype allele was identified by a 700 bp and heterozygous with double bands with 700bp and 734bp.

Genotyping of all other transgenic animals used in this thesis was performed by the laboratories providing the samples and encoded to ensure a blind analysis in order to avoid bias.

2.3 Perfusions

Animals were anesthetized by injecting 25 µl euthatal (90mg/kg). Following a negative response to a reaction test the animal was placed in the operating table, in the spine position, and fixed with needles to a cork board. Ear or tail biopsies were collected for genotyping. Access to the heart was achieved by cutting through the rib cage from the diaphragm. Intracardial perfusion was achieved by insertion of a butterfly needle into the left ventricle, and pumped with 0.1M phosphate buffer in order to remove all blood from the body. With the aid of forceps the right atrium was

nicked to allow ensanguination, followed by perfusion of 50ml per animal of fixative solution (4% paraformaldehyde and 3% glutaraldehyde in 0.1M phosphate). The tissue of interest was excised following perfusion and emerged in the same fixative solution overnight at 4ºC. The following morning the tissue was rinsed twice in 0.1M PB and kept immerged in phosphate buffer at 4ºC until processing for electron microscopy.

All perfusions of dominant negative β1 integrin, dominant negative β3 integrin and dominant negative β1 integrin // neuregulin 1 type III $^{+/-}$ mice was performed by the author of this thesis. All other transgenic animals used were perfused by the laboratories of the collaborators where these mice were housed. In the cases where the author did not perform the perfusions, the head of the animals was sent to the author for dissection and subsequent tissue processing and analysis.

2.4 Embedding of samples

Following fixation, tissue was incubated in 1% osmium tetroxide overnight at 4ºC in a dark plastic container.
Tissues were dehydrated in a succession of 15 second baths of crescent concentrations of ethanol (70%, 90%, 3 times 100%). Following dehydration, the samples were immerged twice in propylene oxide for 15 seconds, followed by an overnight incubation in 50/50 propylene oxide/resine TAAB. Embed in new TAAB resine mix the following day. Samples dry at 60ºC overnight.

Embedding of samples was performed by the Department of Anatomy, University of Cambridge, UK.

2.5 Electron microscopy

The embedded samples were cut by the author of this thesis in the Department of Anatomy, University of Cambridge, and prepared using a Leica ultra-cut UCT microtome. Semi-thin sections were stained with methylene blue. Ultra-thin sections were cut and stained with saturated uranyl acetate in 50% ethanol and lead citrate, and ready to the electron microscope. The ultra-thin sections were examined in a Philips CM100 transmission electron microscope.

The sections results were captured with a 3.90k magnification, and 10 to 12 different non-overlapping regions were chosen to take photos.

2.6 Computational analyses

G-ratio and percentage of myelinated axons measurements involve drawing the axon outline, and the axon outline with myelin. These were performed with Openlab image analysis software (Improvision). Excel (Microsoft) was used to calculate the axon, and axon with myelin, radius or diameter.

The g-ratio measurements are the ratio between the axon radius and the axon radius with the myelin. The number of axons drawn was at least 100, from 5 or more non-overlapping images for each sample.

The percentage of myelinated axons was obtained by drawing the outline of all myelinated and unmyelinated axons. The number of axons drawn was at least 300, from 3 or more non-overlapping images for each sample.

All computational analyses was performed by the author of this thesis.

2.7. Immunohistochemistry

For imaging purposes full brains were perfused in 0.4% paraformaldehyde in 0.1M phosphate buffer, post-fixed over night and snap-freezed the following day. Sections were obtained using a cryostat and tissue morphology was assessed by H&E (performed at the QMRI automated service – Shandon Varistain Gemini ES) and luxol fast blue.

2.7.1 Snap Freezing Brains

Following perfusion and dissection of the brain, brain was post fixed (4% PFA) overnight at 4ªC. The next day brains were placed in 30% sucrose solution (the brain should float) at 4ºC. Once brain had sank (usually within 24h), brains were placed over a paper towel to blot the excess sucrose solution.

Brains were placed in a small Petri dish and covered with OCT embedding matrix. A clean cut was performed to divide the brain using a razor blade. Brains were snap frozen in ice cold acetone, previously placed in dry ice, and left for 20 seconds. Frozen brains were stored at -20ºC for 24h followed by storage at -80ºC.

All snap freezing was performed by the author of this thesis.

2.7.2 Cryostat

10 μm sections were cut from the frozen blocks and mounted onto Superfrost plus slides, previously keep at room temperature.

All cryostat sectioning was carried through by the author of this thesis.

2.7.3 Haemotoxylin and Eosin

Haemotoxylin and eosin (H&E) was performed in the Shandon Varistain Gemini ES, from Thermo Fisher according to manufactures instructions. Briefly, a pre-stain step of a 3 cycles of xylene (2 minutes, 1 minute, 1 minute) was followed by 3 cycles of alcohol (1 minute each) and rinsed with water (1 minute). Heamotoxilyn was added (4 minutes), rinsed with water (1 minute), followed by acid alcohol (15 seconds), rinsed with water (1 minute), blueing agent (1 minute), rinsed with water (1 minute), aqueous eosin (1 minute), rinsed with water (1 minute). A post-stain step followed with 3 cycles of alcohol (1 minute each) and 3 cycles of xylene (1 minute each).

H&E staining was performed by the Histology Department, QMRI, Edinburgh, UK.

2.7.4 Luxol Fast Blue

Sections were stain in Luxol fast blue overnight and washed in water in the following morning. Sections were incubated in a 0.1% lithium carbonate bath for 5 for seconds, followed by 70% alcohol for 20-30 seconds. Rinsed with water for 20 seconds and counterstained in 0.1% cresyl violet for 10 minutes in 1% acetic acid. Sections were dehydrated in a crescent concentration of ethanol baths followed by clearing and mounting.

Luxol fast blue staining was performed by the Histology Department, QMRI, Edinburgh, UK.

2.8 Myelinating co-cultures

Dorsal Root Ganglia (DRGs) were dissected from E14 – E16 rats and digested for 50 minutes at 37ºC with 1.2 U/ml Papain (Worthington), 0.24 mg/ml L-cystein (Sigma-Aldrich), and 40 μg/ml Dnase (Sigma-Aldrich). Papain solution was previously incubated for 10 minutes at 37ºC to ensure papain full activation. DRGs were plated onto 22 mm coverslips pre-coated with poly-D-lysine (10 μg/ml, Sigma) and growth factor reduced matrigel (1:40 dilution, BD Biosciences) at a density of 15 x 10^4 cells/ml.

Neurons were grown for 17 days in dulbecco's modified eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FCS, Gibco) and nerve growth gactor (NGF, 100 ng/ml, AbD Sevote). Cultures were pulsed with intercalated growth (NGF) and anti-mitotic (fluorodeoxyuridine – Fdu, 10 μM, Sigma) factors.

Oligodendrocyte precursor cells (OPCs) were obtained from forebrains of P0 to P2 rats. Cerebral cortices were dissected and meninges removed, transferred to a small vial with 1ml of minimum essential medium (MEM, Gibco), triturated and dissociated with 1.2 U/ml Papain (Worthington), 0.24 mg/ml L-cystein (Sigma-Aldrich), and 40 μg/ml Dnase (Sigma-Aldrich). Papain solution was previously incubated for 10 minutes at 37ºC to ensure papain full activation. Dissociated cells were plated on poly-D-Lysine (10 μg/ml, Sigma) coated T75 tissue culture flasks and grown at 37ºC in DMEM (Gibco) supplemented with FCS (Gibco).

After 10 days the OPC cultures were shaken at 250 rpm for 1 hour at 37ºC to remove loosely adherent microglia and debris. Medium was change, and cultures were then shaken again overnight (250 rpm, 37ºC) to detach the top layer of OPCs and remaining glia. Incubating cells in bacteriological plastic dishes (Sterilin) for 20 – 25 minutes at 37ºC removes microglia.

OPCs were added to each coverslip with purified neurons at a density of 15 x 10^4 cells/ml in myelination medium: 250ml Neurobasal medium, 250ml of DMEM (high glucose), add 5ml Glutamine, 5ml Pen/Strep, 5ml insulin and transferase supplement, 5ml of 10x Sato, 10ml B27, 500 µl NAC and 500 µl D-biotin 1000x. Made up in filter unit.

Co-cultures were maintained for 14 days, with fresh medium added every 2 days. Co-cultures were treated with CD29 antibody (β1 integrin blocking antibody, BD Pharmigen) and an IgM control antibody (λ1 Monoclonal Immunoglobulin Isotype Standard, BD Pharmigen). Different coverslips received antibody at different time points. These antibodies were added either at same time as OPCs (T=0), one (T=1) or three (T=3) days after adding OPCs.

All *in vitro* work was performed by the author of this thesis.

2.8.1 Immunocytochemistry

Cultures were fixed with 4% paraformaldehyde (PFA) for 10 – 15 minutes at RT, blocked for 20 – 30 minutes with blocking solution (PBS containing 40% Goat Serum (Sigma) and 0.4% Triton at RT. Rinsed with PBS, followed by incubation of 1.30 – 2 hours with primary antibodies: rat anti-MBP (1:100), rabbit anti-NG2 (1:200) and chicken anti-neurofilament (1:5000). After rinsing with PBS cells were incubated for 1.30 – 2 hours with secondary antibodies: MBP (Alexa 488 anti-rat antibody; 1:500), NG2 (Alexa 555 anti-rabbit antibody; 1:500) and neurofilament (Amca anti-chicken; 1:100). After secondary antibodies, coverslips were rinsed again with PBS and carefully placed in the middle of Superfrost Plus glass slides, with a drop of Flourmount-G.

All immunocytochemistry was executed by the author of this thesis.

2.8.2 Confocal microscopy and quantification

10 to 12 pictures with a 20x objective for MBP, NG2 and neurofilament staining were taken in the Leica Confocal Microscope. For each coverslip, myelination was quantified by counting the number of myelinating MBP positive oligodendrocytes as a percentage of the total number of MBP positive oligodendrocytes.

All confocal imaging was performed by the author of this thesis.

2.9 Statistical Analysis

Statistical analysis was performed using the GraphPad Prism software (GraphPad Software Inc.). Data shows mean ± SEM. G-ratio analysis using Student's *t* test; percentage of myelination per axon diameter analysis using two-way ANOVA; average axon diameter and percentage of unmyelinated axons analysis using Student's *t* test; percentage of dysmyelination analysis using Wilcoxon signed-rank test. n represents the number of axons quantified for any given experiment.

Chapter 3

Role of β1 integrin in central nervous system myelination

Chapter 3

Role of β1 integrin in central nervous system myelination

3.1. Introduction

Oligodendrocytes in the central nervous system (CNS) and Schwann cells in the peripheral nervous system (PNS) go through a cytoskeleton morphological change to produce myelin, a lipidic membrane with insulating properties that facilitates efficient saltatory conduction and therefore a faster propagation of the nervous impulse. The signals that mediate this process have long been researched and one of the potential regulators of myelination is a major family of extracellular matrix (ECM) receptors: integrins.

Central hypothesis

The central hypothesis is that integrins contribute to the axoglial signals regulating the initiation of myelination, Since most of the available data in the literature was performed *in vitro* (β1 integrin knockout mice are embryonically lethal) a new transgenic approach of a dominant negative β1 integrin could shed light into the role of integrins in myelination.

Statement of aims

1. To characterize myelination of dominant negative β1 integrin mice by analysing the g-ratio, percentage of myelination and percentage of unmyelinated axons by electron microscopy at different time points and different brain structures.

2. To quantify the percentage of oligodendrocytes in co-cultures in the presence of β1 integrin blocking antibody.
3. To characterize myelination of dominant negative β3 integrin mice, used as a control for the transgenic approach for the generation of dominant negative β1 integrin mice, by analysing the percentage of myelination and percentage of unmyelinated axons by electron microscopy at different time points.
4. To characterize myelination of the β1 integrin knockout mice, used as a control for the different expression of β1 integrin, by analysing the percentage of myelination and percentage of unmyelinated axons by electron microscopy.
5. To characterize myelination of the focal adhesion kinase knockout mice. Focal adhesion kinase is a downstream signalling molecule from β1 integrin that should replicate the same phenotype as the dominant negative β1 integrin mice, by analysing the g-ratio, percentage of myelination and percentage of unmyelinated axons by electron microscopy at different time points.
6. To analyse myelin aberration in the focal adhesion kinase knockout mice by electron microscopy.

3.1.1 Integrins

Integrins are the most abundantly expressed family of ECM-binding receptors and are involved in the regulation of many fundamental cellular functions such as proliferation, migration and survival (reviewed by ffrench-Constant, 2004).

The term integrin was originally coined in 1987 to describe an emerging family of integral membrane proteins linking ECM to the cytoskeleton (Hynes, 1987). The term "integrin" is representative of their integrating function since they can bind cytoplasmic proteins, ECM ligands and soluble ligands (reviewed by Takada *et al.*, 2007) initiating and regulating several intracellular signalling pathways (Giancotti *et al.*, 1999; Hynes, 2004; Schwartz, 2011).

Integrins are only present in metazoans and are conserved throughout different species such as mammals, chicken, zebrafish, *Drosophila melanogaster* and *C. elegans*, which has proven to be very important in understanding the function of many integrins (reviewed by Takada *et al.,* 2007).

3.1.2 Integrin structure and activation

Integrins form a highly conserved family of heterodimeric transmembrane receptors present in all metazoans (Arnaout *et al.,* 2005), comprising two non-covalently associated subunits: α and β, each encoded by separate genes. In mammals there are 18 α subunits and 8 β subunits which can heterodimerize into 24 different integrin receptors, which vary in their ligand binding, cellular functions and expression patterns (Fig. 3.1B) (Hynes *et al.,* 2000; Hynes, 2002).

The α and β subunits that form the integrin receptor are type 1 glycoproteins characterized by a large cellular domain, a single transmembrane domain and a short cytoplasmic domain. The large transmembrane domain is responsible for ligand binding and comprises up to 1104 residues for the α subunits, whilst the β subunit has up to 778 residues. The single-spanning α-helical transmembrane domain is formed from 25 to 29 amino acids, and the short cytoplasmic domain which spans from 20-40 amino acids, to 45-60 amino acids for the α and β subunits, respectively (Hynes, 2002). β4 integrin is an exception as it binds to intermediate filaments instead of the actin filaments and therefore its cytoplasmic domain is composed of 1018 residues (de Pereda *et al.,* 1999). Half of the α subunits have a von Willebrand Factor type A domain (I/A), an additional extracellular domain of 190 amino acids that is the major site of ligand binding (Arnaout *et al.,* 2005).

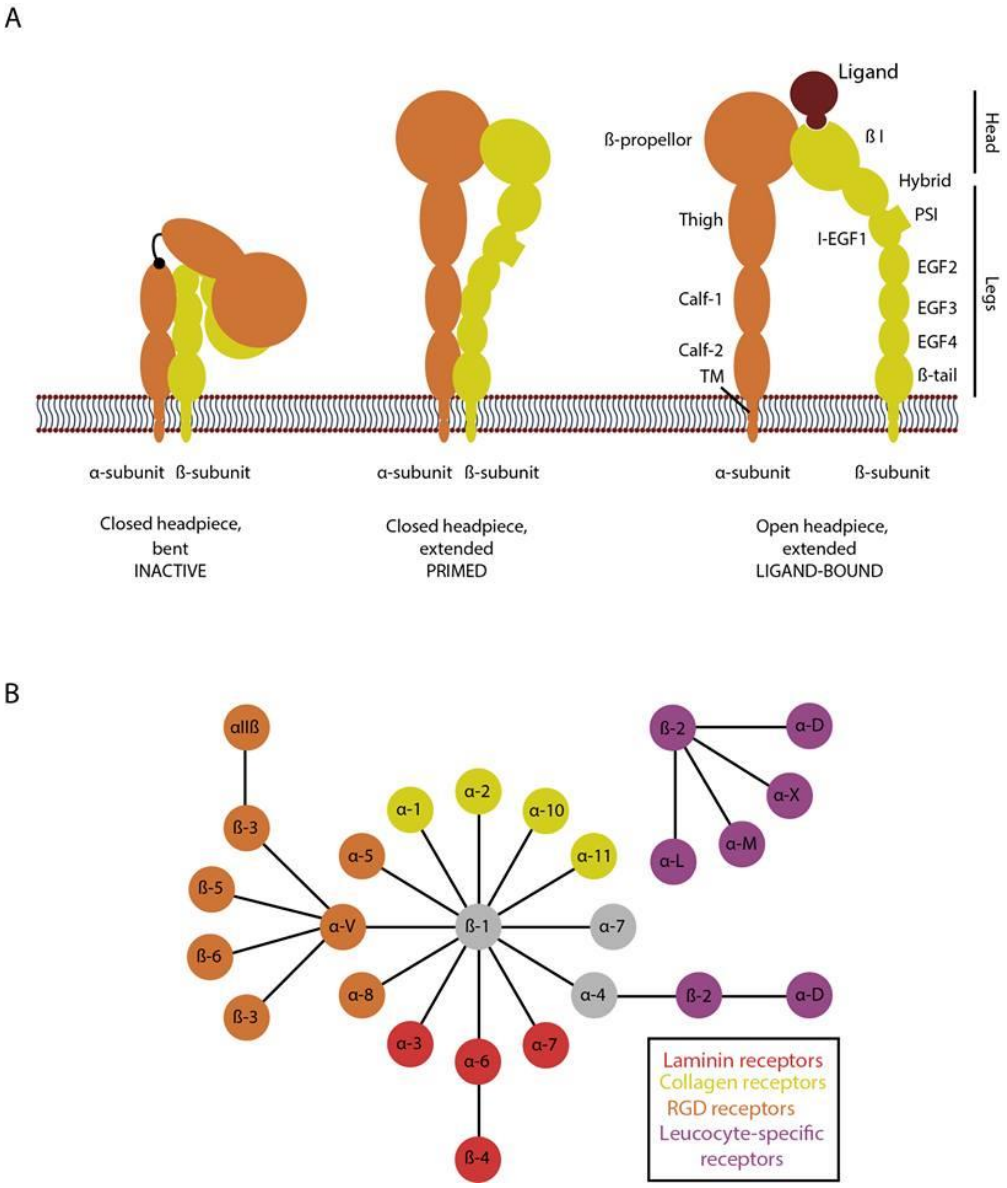


Figure 3.1 – Integrin activation and their ligands. Integrins comprise an α and β subunit that can heterodimerize and are activated by ligand binding to extracellular matrix molecules. (A) Schematic representation of the three conformational states of integrins (inactive, primed and active) which exist in a dynamic equilibrium (Adapted from Luo *et al.*, 2007). (B) The combination of the 18α and 8β subunits generates 24 known mammalian integrins, with differing ligand specificity (Adapted from Hynes, 2002).

Xiong and co-workers published the crystal structure of the extracellular domain of the αvβ3 integrin (an I/A-lacking domain integrin) in its unligated form (Xiong *et al.*,

2001) and ligated form with a RGD peptide (Xiong *et al.,* 2002) showing that the α (β propeller) and the β (βI domain) subunits establish contact to form the ligand binding "head" region. This forms a bent conformation resulting in a low affinity state. The "head" region comprises two legs each with four domains. The α subunit leg comprises a thigh, genu/knee, calf1 and calf2 domains while the β subunit leg includes an immunoglobulin-like hybrid domain, a N-terminal plexin-semaphorin-integrin (PSI) domain, four epidermal growth-factor (EGF)-like repeats and a β tail domain (βTD) (Fig. 3.1A). This allows integrins to adopt different conformational states of activation with different affinity levels; these are thought to be modulated either by their ligands or intracellular signals (Lou, 2006).

Integrins can have three different conformational states: inactive, primed and active (Fig. 3.1A). The *inactive* state is characterised by its bent conformation and closed head piece and it is considered a low affinity state (Shimaoka *et al.,* 2000). Although it is termed inactive, it is known to bind ligands such as fibronectin fragments (Adair *et al.,* 2005). By adding Mn^{2+} or RGD peptide (ligand mimetic peptides), the integrin structure is extended and forms the two other known conformational states. The *primed* state (also termed intermediate stage) has a closed head piece and extended conformation and corresponds to an activated state with intermediate affinity. Finally the *active* state consists of an extended conformation with the head piece open and the presence of a ligand and corresponds to the highest affinity state (reviewed by Takagi and Springer, 2002).

These conformational states exist in equilibrium and one of the models proposed to explain the conformational and activation changes is the hybrid-domain swing-out model. This model was based on observations made through electron microscopy by comparing the structure of the inactive αvβ3 and the active αIIbβ3 integrins. In these structures the β subunit hybrid domain undergoes a 60º shift, generating the separation of the α and β subunit knees (genus). It is considered that the β1 domain α7 helix undergoes a downward displacement due to this swing, which in turn is responsible for allowing accessibility to the β1 domain metal-ion-dependent adhesion site (MIDAS), which increases ligand affinity. Ligand binding stabilizes the

structural extension, but extension *per se* is not enough to increase affinity, a higher ligand-binding affinity results from conformational changes in the head piece (Xiao *et al.,* 2004). The two transmembrane domains separate upon ligand binding (Luo *et al.,* 2004). This activation model is specific for extended conformations, but an alternative deadbolt model has been suggested, since there is evidence that integrins are still able to bind ligands when in a bent structure (Adair *et al.,* 2005; Arnaout *et al.,* 2007; Xiong *et al.,* 2003; Xiong *et al.,* 2002).

Integrin activation and function also depends on the short cytoplasmic domain which has conserved amino acid sequences both in the α and β subunit. These conserved amino acids are the NPxY motif in the β subunit and membrane proximal **GFFKR** (in the α subunit) and **LL**V-I**HDR (**in the β subunit) (Fig. 3.2). Integrins can be activated by mutations on the membrane proximal highly conserved regions, achieved by mutation on the arginine 935 (R in the GFFKR sequence) of the αIIb subunit or mutation of the aspartate 723 (D in the IHDR sequence) of the β3 subunit (Hughes *et al.,* 1996; O'Toole *et al.,* 1994). Interestingly, mutations converting arginine 935 into aspartate, and the aspartate 732 into arginine do not affect integrin activation suggesting the presence of a salt bridge between the α and β subunits that preserves the integrin inactive state (Hughes *et al.,* 1996). Vinogradova and co-workers (2002) showed by nuclear magnetic resonance imaging an interaction between the membrane proximal regions of the α and β subunit during their study of the structural mechanisms of αIIbβ3 integrin activation. In this study it is described how αIIbβ3 integrin can be activated by disrupting this short bridge through point mutations or interactions with the cytoplasmic protein talin (Vinogradova *et al.,* 2002). Takagi and co-workers showed that in the α5β1 integrin, the separation of the cytoplasmic domains can be achieved by the unclasping of the membrane proximal regions suggesting that separation of the cytoplasmic domains might be a mechanism for either initiation of inside-out signalling or a late stage of outside-in signalling (Takagi *et al.,* 2001).

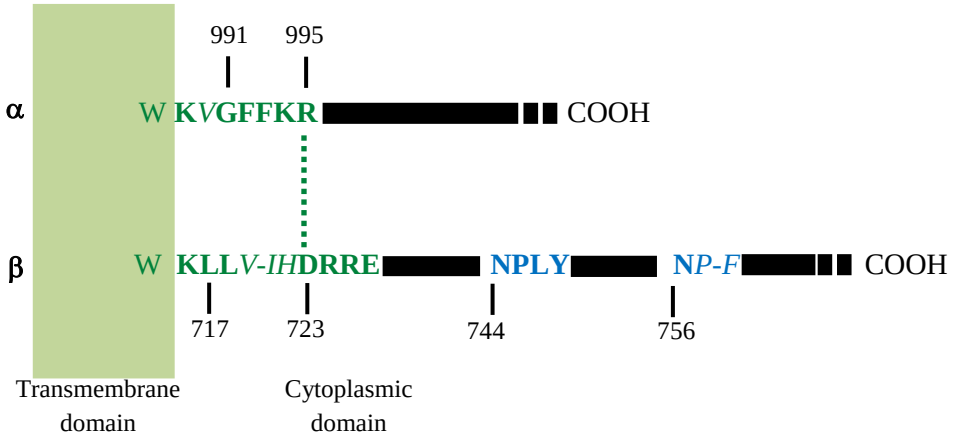


Figure 3.2 – Schematic representation of the conserved domains and respective amino acid sequences of the α and β subunit cytoplasmic tails. Bold letters represent near-invariant residues, italic letters represent residues conserved in at least 3 integrin subunits and dashes represent non-conserved residues. The dotted line represents the salt bridge between R995 and D723. NPxY motifs are in blue. Adapted from Hynes, 2002 and Liu *et al.*, 2000.

3.1.3 Integrin bidirectional signalling

The role of integrins in adhesion is characterized by the unusual feature of bidirectional signalling across the plasma membrane. This bidirectional signalling links the ECM outside the cell and the cytoskeleton inside the cell (Hynes, 2002; Legate *et al.*, 2009). *Inside-out* signalling (also referred to as upstream signalling or integrin activation) is characterized by the regulation of extracellular binding affinity by intracellular signals. O*utside-in* signalling (or downstream signalling) is associated with ECM binding to integrin that modulates their intracellular signalling pathways (Giancotti and Ruoslahti, 1999).

Inside-out signalling

Inside-out activation of integrins relies on the separation of the cytoplasmic and transmembrane regions of the α and β subunits (Kim *et al.*, 2003; Luo *et al.*, 2004; Vinogradova *et al.*, 2002). This separation leads to a conformational change of the integrins' structure which goes from a bent to an extended conformation increasing

their ligand-binding affinity (Luo and Springer, 2006; Takagi and Springer, 2002). Work done by O'Toole and co-workers where either the full or part of the cytoplasmic domain was deleted, resulted in a constitutively active integrin (O'Toole *et al.,* 1994; O'Toole *et al.,* 1991).

Integrins can be activated by different cytoplasmic proteins that bind to the α and β subunits such as calcium integrin binding protein (CIB), cytohesin-1, β3-endonexin, talin and kindlin-2 (Bledzka *et al.,* 2012; Calderwood, 2004; Hughes and Pfaff, 1998).

The current model of integrin activation is based on talin. Talin is a cytoskeletal protein that comprises a C-terminal rod domain (responsible for dimer formation and encloses binding sites for vinculin and actin) and an N-terminal FERM (F for 4.1 protein, E for ezrin, R for radixin and M for moesin) domain (also termed the head region) comprising the main binding sites for focal adhesion kinase (FAK) and phosphatidyl inositol biphosphate (PIP2) (Nayal *et al.*, 2004; Wegener *et al.,* 2007). The FERM domain comprises three subdomains: F1, F2 and F3. The F3 domain itself is sufficient to activate integrin, since it is a phosphotyrosine binding (PTB)-like domain with high affinity binding properties to the β subunit (Wegener *et al.,* 2007). Binding of the PIP2 to talin results in the exposure of the PTB domain in the head region of talin, which binds the membrane proximal NPxY motif of the β integrin cytoplasmic domain (Garcia-Alvarez *et al.,* 2003). This talin head interaction with the integrin β-tail disrupts the salt bridge between the α and β subunits, known to stabilise the bent conformation (Fig. 3.3) (Hughes *et al.,* 1996; Kim *et al.,* 2003; Wegener *et al.,* 2007). The binding of talin to the β subunit is a common final step of integrin activation (Tadokoro *et al.,* 2003; Tanentzapf and Brown, 2006). Recently, kindlin-2 was described to function in a similar way to talin to activate integrins. Like talin, kindling-2 also has a FERM domain and interacts with the β1 and β3 cytoplasmic tails (whilst talin interacts with β1, β2 and β3 subunits). Bledzka and co-workers showed that although talin and kindling-2 do not interact, they can act simultaneously at the β3 tail suggesting a cooperating mechanism for integrin activation (Bledzka *et al.,* 2012).

Inside-out signalling in integrins has been associated with three major signalling pathways: PKC, PI3K and Ras superfamily of small GTPases pathways.

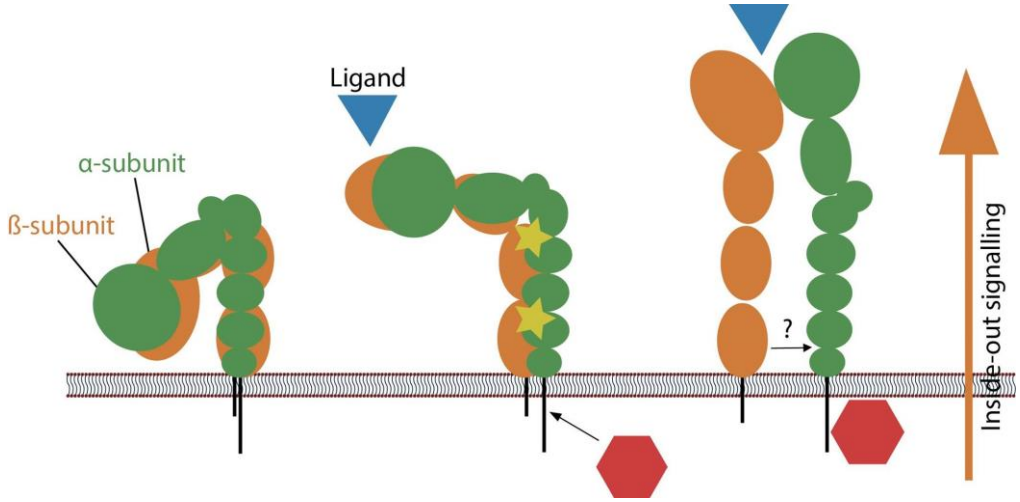


Figure 3.3 - Integrin inside-out signalling. Upstream signalling of integrins is dependent of a ligand, this will lead to a disruption of the non-covalent clasp between both subunits and a subsequent binding of talin (hexagonal shape) which lead to a conformational change from a bent state to a constitutive activation of the integrin receptor. α subunit is in brown, β subunit is in green. Adapted from Askari *et al.,* 2009.

Outside-in signalling

Integrin inside-out activation increases the affinity binding of specific integrins to ECM ligands, but the avidity of this interaction needs to be increased to allow stronger cellular adhesion; this is accomplished by integrin clustering. The clustering of integrins allows a high number of weak interactions (in the order of hundreds or thousands) to increment the signalling and form a tight and strong adhesion unit. This increased avidity is a result of collaborative effects to resist bond deterioration (Yu *et al.*, 2010). Initially integrins cluster into nascent adhesions (Choi *et al.*, 2008), a portion of these nascent adhesions follow on maturation and became focal adhesions, and lastly fibrillar adhesions and podosomes (Geiger *et al.*, 2001; Lock *et al.*, 2008).These complexes are responsible for integrin outside-in signalling (Legate *et al.*, 2009).

Integrins modulate different cellular functions such as: cell shape, migration, differentiation, growth and survival (Grashoff *et al.,* 2004; Legate *et al.,* 2009; Ridley *et al.,* 2003; Shattil and Newman, 2004). Activation of integrins depends of extracellular ligands, although interaction with transmembrane and cytoskeletal proteins can also have the same function. Binding of such proteins to these adhesion complexes modulates different pathways such as the extracellular signal regulated kinase/mitogen-activated protein kinase (ERK/MAPK), phosphatidylinositol 3-kiase (PI3K), Akt/protein kinase B (PTB), Jun-amino terminal kinase/JUN (JNK/JUN), and nucleofactor kB (NF-kB).

The short integrin cytoplasmic tails lack enzymatic activity, so integrins depend on the recruitment of adaptor molecules and signalling proteins (such as talin and vinculin) which are responsible for the interactions between integrins and cytoplasmic proteins like FAK, Src, ILK and paxilin, in order to reorganize the cytoskeleton and provide catalytic activity to the focal adhesion (Wiesner *et al.,* 2005). The interaction of integrins with the actin cytoskeleton occurs via adaptor proteins. Talin binding to the β subunit and the actin cytoskeleton is essential for the initial integrin-cytoskeleton association (Priddle *et al.,* 1998; Zaidel-Bar *et al.,* 2003; Zhang *et al.,* 2008). Talin binding is followed by a conformational change that exposes the vinculin binding and dimerisation sites. Binding of vinculin to the nascent adhesion stabilises the talin-actin bond (Gallant *et al.,* 2005; Humphries *et al.,* 2007). Other proteins are known to also be recruited for the stability of this interaction such as α-actinin, a binding partner of talin and vinculin; integrin-linked kinase (ILK) that binds to actin via parvin; focal adhesion kinase (FAK); kindlins, which act in concert with talin; paxillin, with an early recruitment to the cell-matrix adhesion, tensin and zyxin both being involved in late stages (Legate *et al.,* 2009). α-actinin also regulates maturation and growth of adhesion sites in response to mechanical tension (Fig. 3.4) (Askari *et al.,* 2009).

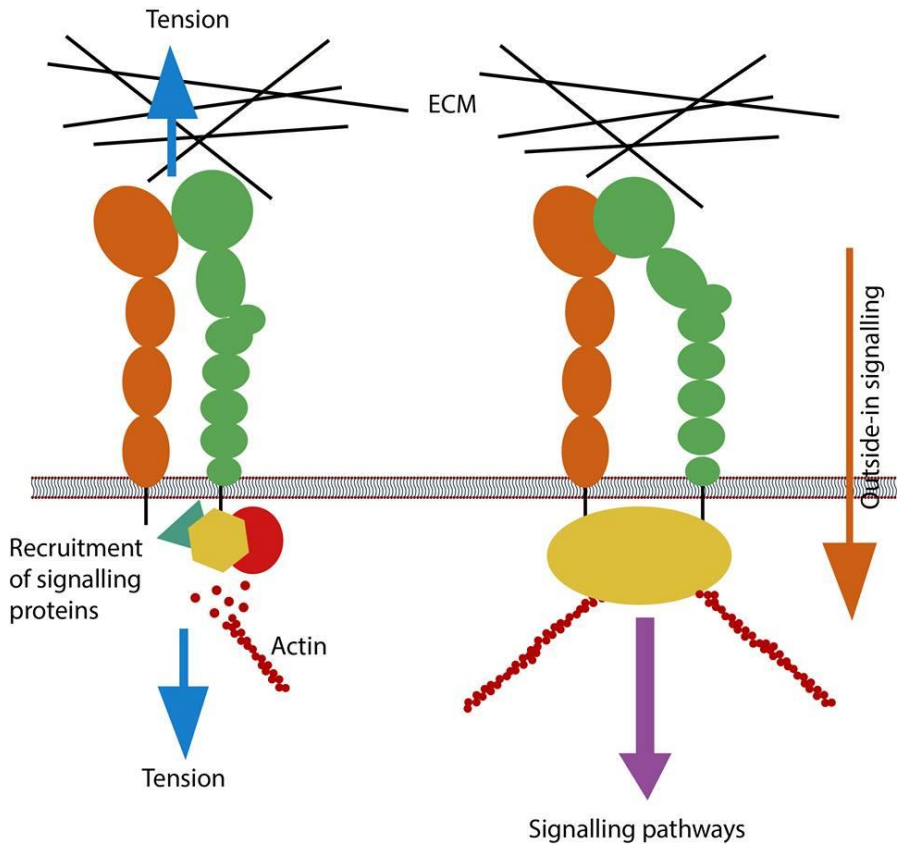


Figure 3.4 – Integrin outside-in signalling. Upon interaction with the ECM and ligand binding (the final step of upstream signalling) there is a recruitment of signalling proteins (such as talin) triggering focal contact formation, this will lead to tension in the integrin receptor, allowing for a further outward movement of the hybrid domain, leading to the activation of the downstream signalling pathways. α subunit is in brown, β subunit is in green . Adapted from Askari *et al.,* 2009.

Integrin downstream signalling pathways

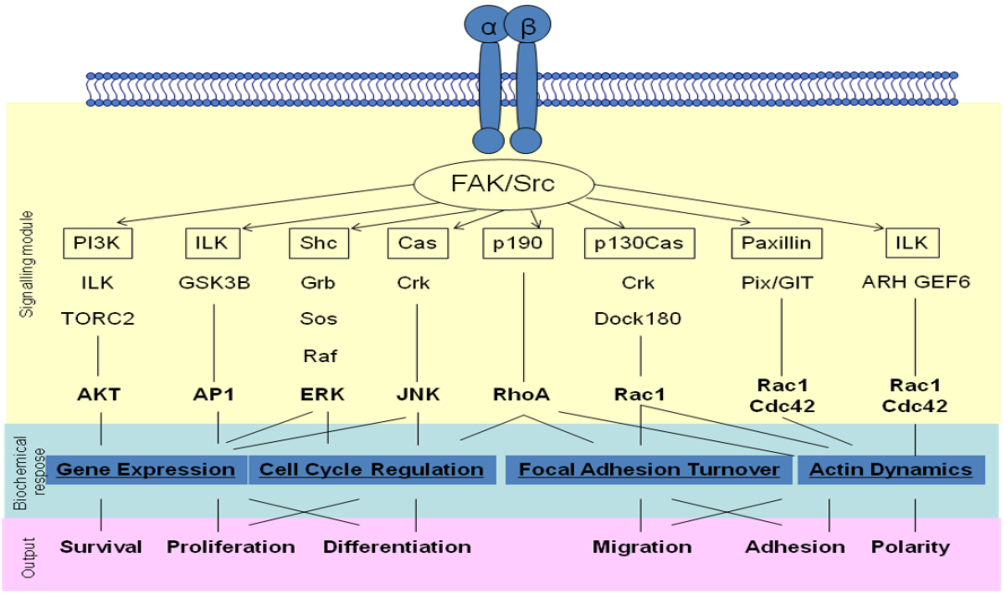


Figure 3.5 – Integrin downstream signalling. Different pathways and respective cellular functions controlled by integrin downstream signalling. Downstream signalling of integrins, through FAK/Src controls different pathways involved in several cellular processes such as survival, proliferation, differentiation, migration, adhesion and polarity, (Adapted from Legate *et al.* 2009)

As previously mentioned, integrins can modulate different cell functions through several signalling pathways (Fig 3.5). Focal adhesion kinase (FAK) is a tyrosine kinase localized at sites of adhesion that can mediate adhesion and signalling through different pathways. FAK is recruited to the nascent focal adhesion through direct interaction with the cytoplasmic tail of the β subunit, or through interaction with cytoskeletal proteins such as talin and paxilin (Giancotti and Ruoslahti, 1999). FAK is a 125 kDa protein that comprises a FERM domain and a focal adhesion targeting (FAT) domain which are responsible for the multiple FAK binding partners that act as effectors of many downstream functions (Mitra *el al.*, 2005). Although FAK is a tyrosine kinase, it acts more like a non-kinase since it lacks the ability to phosphorylate other molecules, with its main role being to recruit other proteins. Instead, FAK can phosphorylate itself or can be phosphorylated by the Src family protein tyrosine kinase (SFKs), which comprise nine members, such as Src, Fyn and Yes (Legato *et al.*, 2009).

Src is a non-receptor tyrosine kinase that constitutively associates with the integrin β subunit (Arias-Salgado *et al.,* 2005; de Virgilio *et al.,* 2004) and forms the Src-FAK complex which is a central point for integrin activation (Martin *et al.,* 2002). Activation of the Src-FAK complex follows integrin ligation and clustering as a result of (1) the dissociation of Csk (an Src inhibitor) from the integrin; (2) transphosphorylation of Src; and/or (3) recruitment of tyrosine phosphatases (such as RPTPα or PTP1B) (Arias-Salgado *et al.,* 2005; den Hertog *et al.,* 1993; Liang *et al.,* 2005; Obergfell *et al.,* 2002; von Wichert *et al.,* 2003). In response to integrin ligation, FAK is activated by autophosphorylation at tyrosine residue 397 (Y397) which is followed by the recruitment of the Src-homology domain (SH) 2 that stabilises the active conformation of Src, which leads to maximal FAK activity by phosphorylation of FAK at Y576, Y577, Y861 and Y925. This results in full activation of both kinases (Schlaepfer *et al.,* 1994; Calalb *et al.,* 1996; Thomas *et al.,* 1998). Phosphorylation at Y416 activates Src, whilst phosphorylation at Y527 has the opposite effect and inhibits Src (Mitra *et al.,* 2006; Schlaepfer and Hunter, 1997). The Src-FAK complex is responsible for recruitment and phosphorylation of different adaptor proteins, such as $p130^{CAS}$ and paxilin that results in the activation of the ERK/MAPK, JNK/Jun and/or NF-kB signalling pathways. Activation of ERK/MAPK pathways by FAK is also achieved by recruiting the growth-factor-receptor-bound-2 (Grb2) and son-of-sevenless (SOS) complex. FAK can also directly activate the Akt/PKB pathway via PI3K and PIP_3 (Legate *et al.,* 2009).

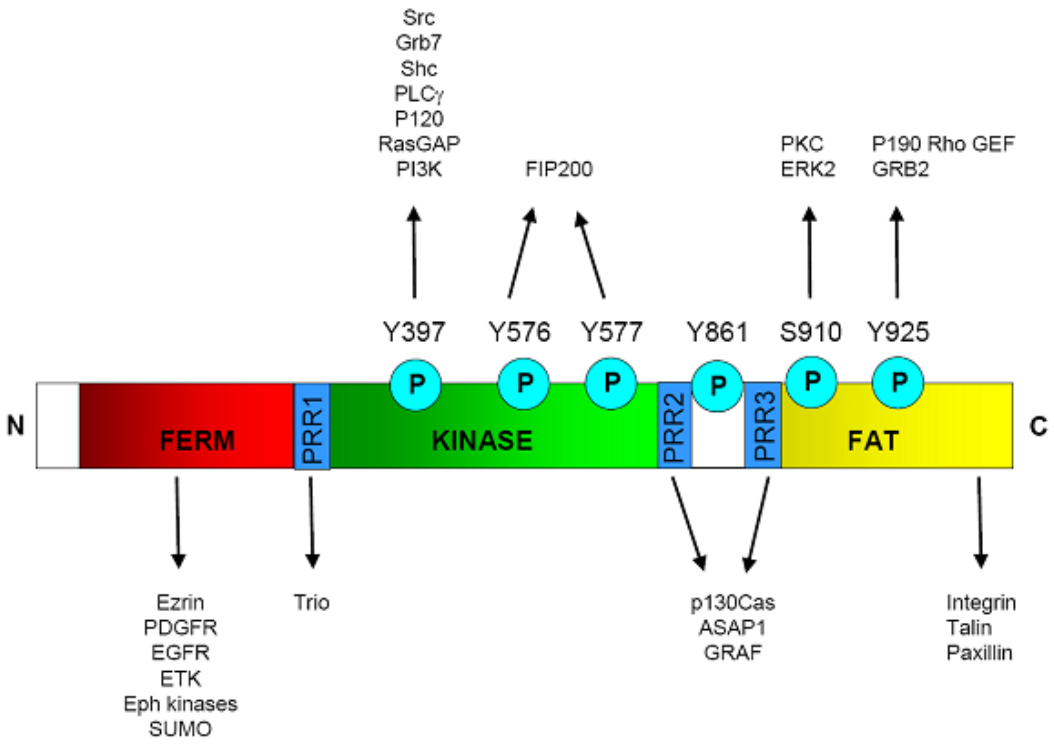


Figure 3.6 - FAK domain structure, phosphorylation sites and protein binding sites. The three domains of FAK: FERM, kinase and FAT. P – represents phosphorylation; arrows - protein binding sites. Adapted from Schwartz, 2001, Mitra *et al.*, 2006, Mitra, *et al.*, 2005, by Dr Colin Chan.

The mitogen activated protein kinase (MAPK) pathway is the major signalling pathway downstream of the Src-FAK complex (Wu *et al.*, 2008), which activates the ERK pathways through integrated signals from Shc, Grb2; SOS and Ras (Danen and Yamada, 2001; Giancotti and Ruoslahti, 1999; Roovers and Assoian, 2000). There are different mechanisms for regulation of the ERK pathway by integrins since ERK is a promiscuous kinase that can phosphorylate more than 100 substrates (Ramos, 2008). One of these mechanisms is through FAK phosphorylation at Y925 via Src, leading to activation of Ras and the downstream ERK pathway (Juliano, 2002). Another mechanism is through activation of caveolin (but does not involve FAK). Grb2 and SOS are recruited by the adaptor protein Shc after its phosphorylation. Shc is phosphorylated after Fyn activation by integrin ligation (Giancotti and Ruoslahti, 1999). Another mechanism is via Shc, which is phosphorylated by Src and FAK.

This leads to ERK translocation to the nucleus followed by gene transcription by phosphorylation of transcription factors (Aplin and Juliano, 2001). The existence of several different mechanisms able to activate downstream pathways shows the complexity of integrin signalling.

FAK activation can also recruit PI3K to adhesion complexes, leading to activation of Akt which regulates cell survival (Chen and Guan, 1994). Akt can be activated by rapamycin (mTOR) or integrin-linked kinase (ILK), depending on the cellular context. ILK lacks kinase activity and binds directly to β1 and β3 integrin and also functions as an adaptor protein which can link integrins to its binding partners (Fukuda *et al.,* 2009). ILK interacts indirectly with actin through parvin, its main binding partner. Cytoskeleton interaction with ILK can also occur via paxilin, which binds parvin and vinculin (Legate *et al.,* 2006). Studies in *C. elegans*, *Drosophila*, mice and zebrafish show that ILK is essential for stabilising integrin-actin interaction (Zervas *et al.*, 2001; Mackinnon *et al.*, 2002; Postel *et al.*, 2008, Wang *et al.*, 2008). Under physiological conditions, mTOR is the main kinase and localizes predominately in the endoplasmic reticulum and the Golgi, and since Akt phosphorylation takes place in the plasma membrane; it is proposed that integrins can switch this pathway on and off since Akt activation through mTOR occurs indirectly via protein phosphatase 2A (PP2A). β1 integrin interacts and activates PP2A, which can dephosphorylate and inactivate Akt. PP2A is also inactivated by mTOR (Peterson *et al.,*1999; Ivaska *et al.,* 2002). It has also been proposed that since ILK binds rictor (an important component of mTOR complex 2), ILK acts as a platform to localize mTOR activity to cell-matrix adhesions (McDonald *et al.,* 2008). Lastly, it is also possible that mTOR and ILK act upstream of Akt via an unknown kinase that would control Akt phosphorylation (Legate *et al.,* 2009).

Another family of enzymes that is linked to outside-in integrin signalling are the GTPases, responsible for a variety of cellular functions such as cytoskeleton organization, transcription factor activity, cell cycle progression, membrane transport pathways, migration and cell adhesion (Schwartz and Shattil, 2000). GTPases have a highly conserved G domain that is hydrolysed through intrinsic GTPase-activity,

modulating GTPase cycling from the active (GTP-bound) to an inactive (GDP-bound) state. The GTPases, cycling from active to inactive state are regulated by guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs) and guanine nucleotide dissociation inhibitors (GDI) (Legate *et al.,* 2009). The GTPase family comprises 5 categories: Ras, Rho, Rab, Arf and Ran (Etienne-Manneville and Hall, 2002).

The initial integrin ligation triggers the activation of Cdc42 and Rac which are involved in cell spreading through formation of actin structures. Cdc42 is known to regulate extension of fillopodia, while Rac is associated with lamellipodia (Burridge and Chrzanowska-Wodnicka, 1996). Cell spreading promotes the upregulation of Rho that induces the formation of actin stress fibres and the maturation of focal adhesions which is followed by an increased FAK activity and paxilin phosphorylation (Ren *et al.,* 1999). Cdc42 is also involved in activation of PI3K, Akt and MAPK pathways (Clark *et al.,* 1998; Yan *et al.,* 1999). Rac has been associated with activation of MAP and JNK (Dolfi *et al.,* 1998). Rho has been involved in activation of MAPK and FAK (Renchaw *et al.,* 1996; Barry *et al.,* 1997).

3.1.4 Integrins and oligodendrocytes

Myelination occurs caudo-rostrally in the brain, and rostro-caudally in the spinal cord. Mouse myelination starts in the spinal cord at birth, with the brain being completely myelinated at P45-60, whereas in humans it starts during the fourteenth week of fetal development, achieving its peak during the first year after birth (Baumann *et al.,* 2001).

Oligodendrocytes are the myelinating cells of the central nervous system (CNS) and express a limited set of integrin heterodimers during development. Oligodendrocyte development comprises a series of overlapping stages, during which the cells induced from the neuroephithelium migrate through the CNS while proliferating, and eventually differentiate, become myelinating cells and either contact an axon and initiate myelination or undergo programmed cell death (Trapp *et al.,* 1997). Raff and

co-workers showed that oligodendrocyte development *in vitro* resembles (in terms of time scale) that observed *in vivo*. This allows for a thorough description of the events involved in oligodendrocyte proliferation, migration and differentiation and for analysing the respective integrin expression pattern during these events through immunoprecipitation methods (Raff *et al.*, 1985; Milner and ffrench-Constant, 1994). From the 24 integrin heterodimers that have been characterized, only 5 of them are expressed in oligodendrocytes: αvβ1, αvβ3, αvβ5, αvβ8 and α6β1. The class of αv integrin receptors are expressed in a sequential manner suggesting a specific role for each of these integrins at the specific time of their expression, with αvβ1 being associated with migration, αvβ3 with proliferation and αvβ5 with differentiation. The two other remaining integrins (αvβ8 and α6β1) are expressed throughout oligodendrocyte development and differentiation (Fig. 3.7) (Milner and ffrench-Constant, 1994; Milner *et al.*, 1997). With an unknown function, the β8 subunit differs from other β-subunits since its cytoplasmic domain is distinct and does not contain any of the known conserved sequences involved in cytoskeletal interaction, such as the NPxY motif (Moyle *et al.*, 1991).

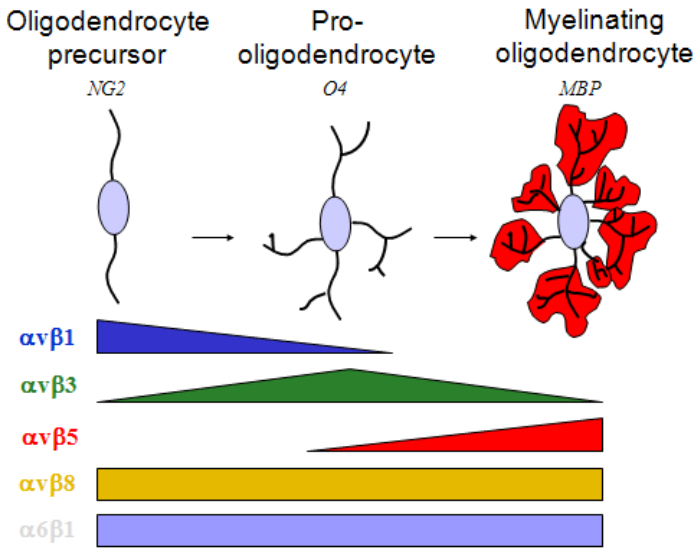


Figure 3.7 – Pattern of integrin expression during oligodendrocyte development. Adapted from http://www.brc.cam.ac.uk/pages/ffrench.html by Dr. Joana Câmara.

Despite the extensive *in vitro* research to identify specific integrins in the different stages of oligodendrocyte development, some issues have arisen. Integrin studies

were performed via immunoprecipitation of biotinylated lysates from non-ionic detergent extraction at 4ºC (Milner and ffrench-Constant, 1994). Microdomains insoluble at 4ºC (termed lipid rafts) have been shown to associate with integrins and growth factors and regulate oligodendrocyte signalling, so it is possible that other integrin heterodimers have been discarded in the insoluble pellet during these experiments, and therefore might be other integrins that have not yet been identified (Baron *et al.,* 2003; Decker *et al.,* 2004; Decker and ffrench-Constant, 2004). Furthermore, most of these *in vitro* findings have not been reproduced *in vivo*. Some integrin knockout transgenic animals with ablation of integrins in the whole organism cause early lethality, i.e. the β1, β8 and αv integrin knockouts (Bader *et al.,* 1998; Fässler and Meyer, 1995; Zhu *et al.,* 2002). Knockout mice for either β3 or β5, or both, survive until adulthood but show no CNS phenotype (Hodilava-Dilke *et al.,* 1999; Reynolds *et al.,* 2002). To gain more insight into the function of integrins in oligodendrocyte development, different approaches, such as conditional mutants, should be examined. Conditional mutants using recombination under the nestin promoter (CNS specific) for β1, β8 and αv integrins have already been reported. In these studies, β1 and β8 conditional knockout did not disturb oligodendrocyte development, but αv conditional knockout showed cerebral haemorrhage and demyelination of the spinal cord and cerebellum (Graus-Porta *et al.,* 2001; McCarty *et al.,* 2005; Proctor *et al.,* 2005). When performing histology in these mice, αvβ8 was only detected in cerebellar and spinal cord axons, and not in oligodendrocytes, suggesting that the phenotype described was due to a loss of αvβ8 integrin that would perturb the axoglial contact (McCarty *et al.,* 2005).

Several *in vitro* and *in vivo* studies have been performed and help to understand the role of integrins during oligodendrocyte development and myelination. A brief overview of these studies will be discussed next.

Integrins in oligodendrocyte development

Oligodendrocytes arise from the neuroepithelium and actively migrate through the CNS to reach the area which they will eventually myelinate. *In vitro*, an agarose

migration assay showed that OPC *migration* is inhibited by blocking antibodies against β1 integrin and RGD peptides (which block αv but not α6 integrins), when in the presence of both PDGF and aphidicholin (a mitotic inhibitor) (Milner *et al.*, 1996; Tiwari-Woodruff *et al.*, 2001). Migration is promoted by fibronectin and laminin and is inhibited by tenascin-C and the integrin ligand collagen. These results show an integrin-dependent mechanism for oligodendrocyte migration (Frost *et al.*, 1996; Milner *et al.*, 1996).

Integrins can regulate growth factor signalling, with PDGF stimulating OPC proliferation through αvβ3 integrin activation (Garcion *et al.*, 2001). Over-expression of αvβ3 integrin in the CG-4 oligodendrocyte cell line promotes proliferation, while both anti-β3 integrin blocking antibodies and β3 integrin dominant negative expression inhibit proliferation *in vitro* (Baron *et al.*, 2002; Blaschuk *et al.*, 2000).

During differentiation oligodendrocyte precursor cells differentiating into myelinating oligodendrocytes show an up regulation of αvβ5 integrin levels (Milner *et al.*, 1994). Furthermore, *in vitro*, CG4 cells supplemented with αvβ5 integrin blocking antibody show a reduction of MBP positive oligodendrocytes. Removal of the antibody leads to a recovering ability of oligodendrocytes to differentiate (Blaschuk *et al.*, 2000). Laminin-α2 and α6β1 integrin stimulate immature oligodendrocyte maturation and the formation of myelin membrane sheets (Colognato *et al.*, 2002).

Oligodendrocytes that fail to establish axonal contact undergo apoptosis (Trapp *et al.*, 1997). Oligodendrocyte survival depends on the presence of axons and is restricted by the availability of axonal-derived survival factors, one such trophic factor is laminin-2 that interacts with integrin receptors expressed on oligodendrocytes (Barres *et al.*, 1992; 1999). *In vitro*, α6β1 integrin contacts axonal laminin and stimulates an increase in oligodendrocyte survival (Barres *et al.*, 1993). Work from our lab showed that oligodendroglial integrins amplify growth factor signalling (specifically, the Scr family kinases) promoting either differentiation and survival, or proliferation (Colognato *et al.*, 2004). Fyn interacts with α6β1 integrin

and is required for amplifying PDGF-mediated survival signalling and promotes myelin sheath formation. Therefore, Fyn regulates both differentiation and survival; Lyn, another member of the Src family kinases is involved in proliferation (Colognato *et al.*, 2004). Another study using oligodendrocytes from transgenic mice lacking the α6 integrin (a laminin receptor), showed a switch in the survival signalling once in contact with axonal laminins (Colognato *et al.*, 2002).

3.2 Results

Myelination in the CNS is a very intricate process and the major players that regulate this process have yet to be identified. Central myelination is performed by oligodendrocytes which after development acquire the capacity to myelinate multiple axons. Different approaches to study oligodendrocyte development and key players involved in myelination have been made throughout the years. In 1980, McCarthy and Vellis described the first protocol for isolation of relatively pure oligodendrocytes (McCarthy and Vellis, 1980). This was a major step towards understanding the myelination process since it was now possible to study oligodendrocyte development *in vitro*. New advances were made with the co-culture of oligodendrocytes with dorsal root ganglion neurons that has been established as an *in vitro* model to study myelination. In these cultures, it is possible to culture axons and glial cells, and track the developmental process of axon ensheathment (Lubetzki *et al.*, 1993). Although these models allowed major breakthroughs in the field, new and more complex systems are needed to fully understand myelination in all its complexion, taking into account not only axoglial interaction but also the full environment that can influence myelination. *Ex-vivo* models have also been described, where slices of tissue are maintained in culture permitting a better overview of myelination, since this culture maintains cell-cell and cell-ECM interactions observed *in vivo*. All of these techniques are crucial for myelination research, but an *in vivo* approach is necessary to fully understand the complexity surrounding myelination, using transgenic animals. Mutant models give us insightful

tips of the molecules regulating different aspects of myelination and associated diseases.

Statistical analysis was performed in all data sets with at least three animals, while for data sets comprising less than three animals only the average g-ratio or percentage of myelination was calculated, since with a low n number of samples no nonparametric test is going to show significance. Average g-ratios and percentage of myelination were given as an indication of trend showing that no abnormalities were observed. The focus of this chapter is the phenotype seen in the optic nerve, an area comprised of small diameter axons, where the effect of the dominant negative β1 integrin was clear in increasing the axon diameter threshold necessary for myelination. Corpus callosum, an area that encompasses both small and large axons was used as a control to confirm the phenotype was specific for small diameter axons. All other structures (ie, cerebellum and spinal cord, characterized by large diameter axons) were included only as exploratory research to confirm these areas do not follow the same trend. Subsequently, this is not critical data for the conclusion of this chapter and does not lessen the conclusions.

This chapter describes the results obtained using different *in vivo* models to understand the process of regulation of myelination.

3.2.1 Analysis of the dominant negative β1 integrin mice

Dominant negative β1 integrin mice were engineered by Dr. Joana Câmara, as previously described (Câmara *et al.*, 2009). In brief, these animals were generated by replacing the β1 integrin extracellular and transmembrane domains with the non-signalling α subunit of the human interleukin-2 receptor (IL2Rα), under the MBP promoter, making it specific to myelinating oligodendrocytes. Due to this change, the integrin intracellular domain is unable to dimerise with an α subunit, bind ligands or cluster with other integrins.

As mentioned in Chapter 1, Friede showed that the ratio between the axon radius and the axon radius plus myelin – termed g-ratio – is suitable to assess normal myelination (Friede, 1972). These mice were characterized throughout development to understand the effect of β1 integrin during myelination. In order to examine myelination, we performed an ultrastructural electron microscopy analysis of different structures of the nervous system. To preserve the integrity of the tissue, mice were intracardially perfused with a solution of glutaraldehyde and paraformaldehyde fixative. Tissue was dissected and processed (by overnight postfixation followed by dehydration and embedding in resin). Ultrathin sections for electron microscopy (prepared in the Department of Anatomy, University of Cambridge, UK) were examined and photographs of non-overlapping regions were taken. For quantification of myelination, these pictures were analysed by drawing the outline of the inner and outer circumferences of the axon (for g-ratio) with a minimum of 100 axons per animal, or the inner circumferences of myelinated and unmyelinated axons with a minimum of 300 axons per animal in at least 3 non-overlapping pictures. This data was generated using Openlab image analysis software (Improvision) and all analyses was done blind, with codes given to each sample only revealed after complete analysis. As exclusion criteria, if the images showed myelin disruption due to inadequate perfusion, that sample would be excluded and not quantified. This allowed for reduction of treatment bias.

In mice, myelination starts around P9, reaches its peak at P17, is complete around P22/23, and by P60 reaches a stable turnover (Wei *et al.*, 2005). In this project we have covered these key time points of myelination.

We have analysed optic nerve and spinal cord of P9-P10 mice. Optic nerve is characterized by small diameter axons and these mice showed no difference between wildtype (g-ratio average = 0.83) and dnβ1 (g-ratio average = 0.80) (Fig. 3.8). Spinal cord is an area with typically larger calibre axons, and consistent with the optic nerve observations, we saw no significant difference between wildtype and dnβ1, with average g-ratios of 0.84 and 0.85, respectively (Fig. 3.9). Only one animal per genotype was analysed in both optic nerve at postnatal day 9 (Fig. 3.8), and spinal

cord at postnatal day P10 (Fig. 3.9) since these were previous samples from Dr. Joana Câmara that were later analysed to verify if these mice showed a transient trend of myelin deficits. No statistical analysis was performed, and comparison was done based on the average g-ratios.

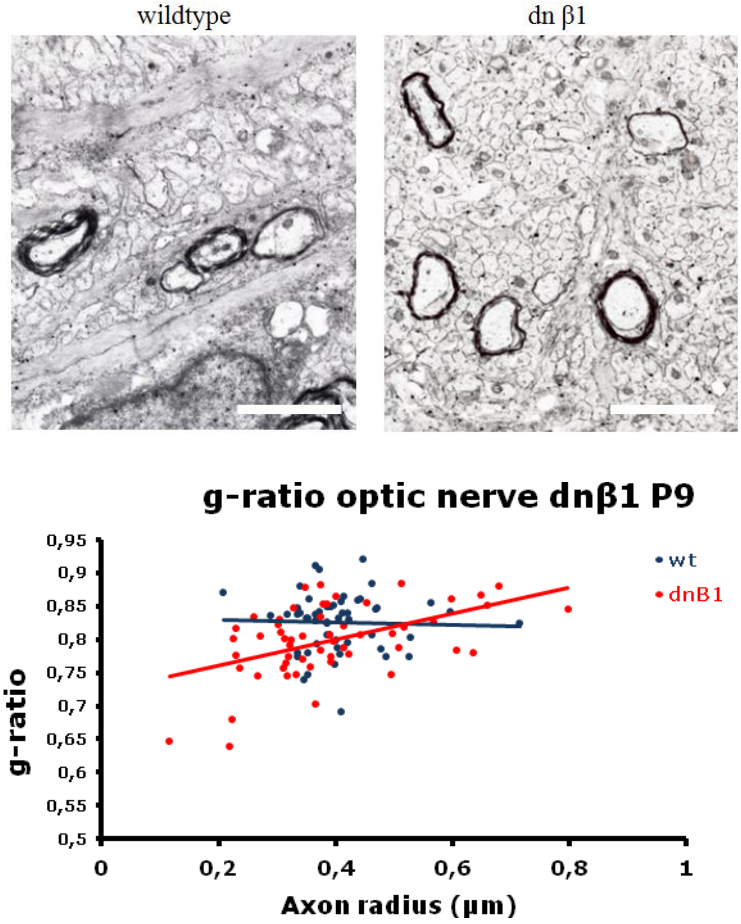


Figure 3.8 – Analysis of optic nerve of dominant negative β1 integrin mice at postnatal day 9. Upper panel: representative electron micrographs of optic nerve of wildtype and dominant negative β1 integrin mice at postnatal day 9. Magnification: 3.90K. Scale bar: 2 µm. Bottom panel: scatter plot displays g-ratios of individual myelinated axons as a function of their respective axon sizes, with the linear regression of the measurements for each animal (1 animal analysed per genotype). Blue circles represent wildtype (n = 55 axons; average g-ratio = 0.83) and red circles represent dominant negative β1 integrin mice (n = 55 axons; average g-ratio = 0.80).

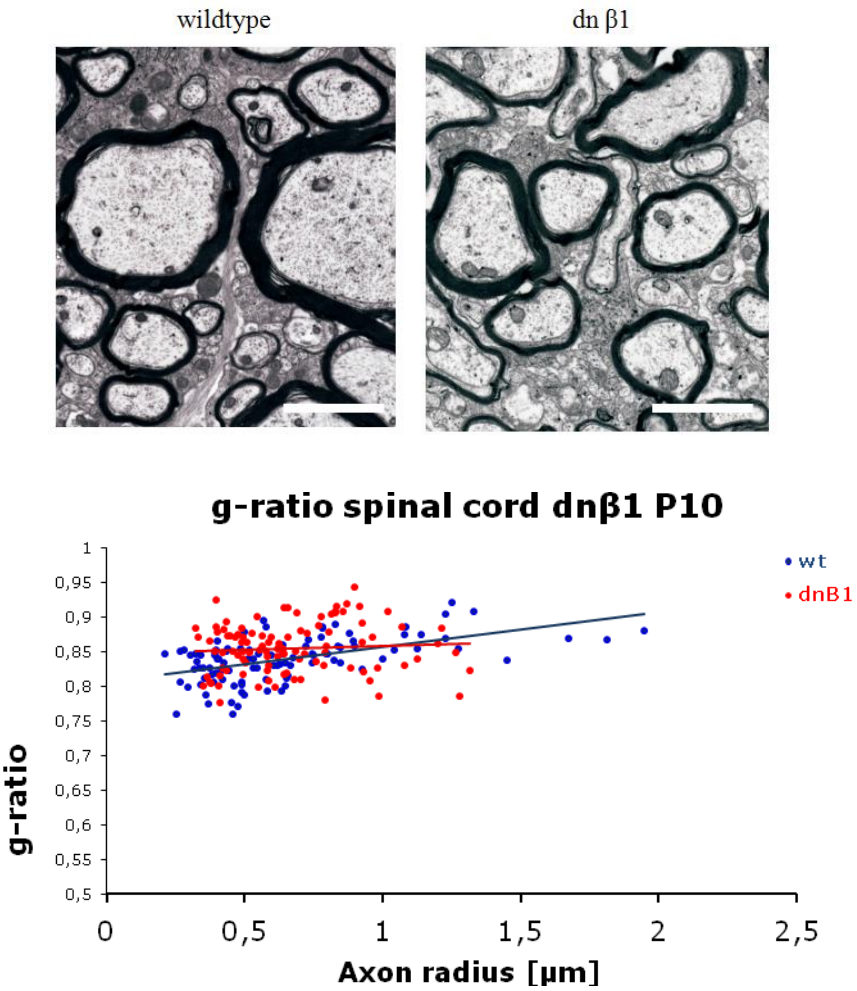


Figure 3.9 – Analysis of spinal cord of dominant negative β1 integrin mice at postnatal day 10. Upper panel: representative electron micrographs of spinal cord of wildtype and dominant negative β1 integrin mice at postnatal day 10. Magnification: 3.90K. Scale bar: 2 µm. Bottom panel: scatter plot displays g-ratios of individual myelinated axons as a function of their respective axon sizes, with the linear regression of the measurements for each animal (1 animal analysed per genotype). Blue circles represent wildtype (n = 100 axons; average g-ratio = 0.84) and red circles represent dominant negative β1 integrin mice (n = 100 axons; average g-ratio = 0.85).

This initial evaluation of CNS myelination in dnβ1 mice showed that early in the process of myelination there is no obvious abnormality in the mutant when compared to the wildtype. Since P9-P10 corresponds to an initial stage of myelination, we analysed these mice at P17, when myelination is not completed but is well underway. We started by comparing the percentage of unmyelinated axons to understand if the mutant mice had any abnormality associated with the decreased β1 integrin

signalling. To quantify the percentage of unmyelinated axons, at least 4 non-overlapping electron microscopic images were analysed, by counting every single axon present and cataloguing it as myelinated or unmyelinated. Analysis of the optic nerve demonstrated no difference (P=0.67) between wildtype (average of percentage of unmyelinated axons = 55.94% ± 6.4, from 3 animals) and dnβ1 (average of percentage of unmyelinated axons = 61.97% ± 11.65, from 3 animals). Both spinal cord and cerebellum showed no difference, with cerebellum wildtype averages of 48.77% and dnβ1 50.0%, from one animal; spinal cord averages of 51.21% and 48.53% for wildtype and dnβ1, respectively, from two animals. After quantifying the optic nerve, cerebellum and spinal cord, there was no discrepancy between these structures when comparing mutant and wildtype animals (Fig. 3.10).

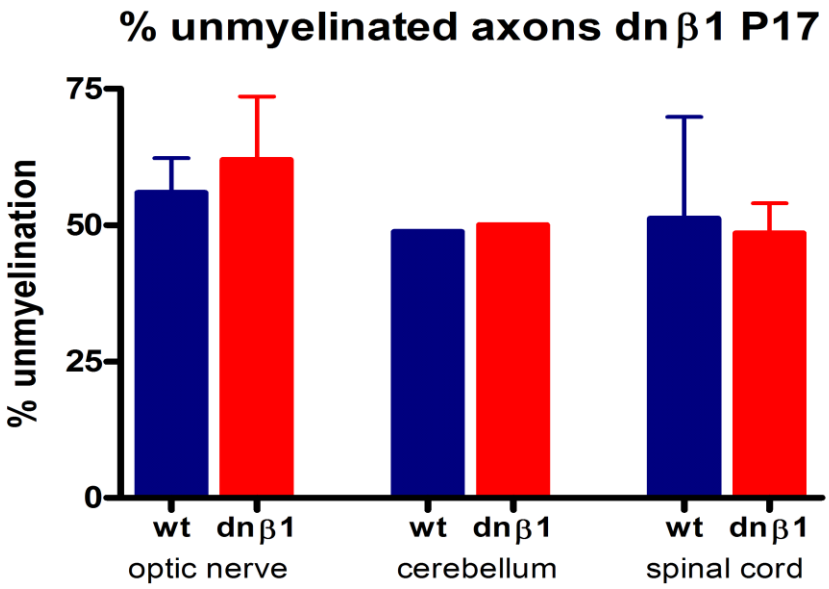
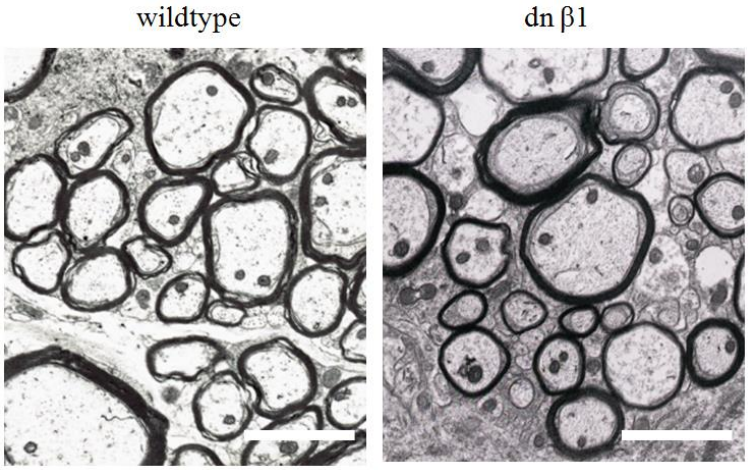


Figure 3.10 – Unaltered percentage of unmyelinated axons of different structures of dominant negative β1 integrin mice at postnatal day 17. The bars represent the average of the percentage of unmyelinated axons. Blue bars represent wildtype animals. Red bars represent dnβ1 animals. In the optic nerve 3 animals were analysed for each genotype with an average of percentage of unmyelinated axons of 55.94% ± 6.4 and for dnβ1 of 61.97% ± 11.65 (P=0.67). In the spinal cord, wildype showed an average of unmyelinated axons of 51.21%, whilst the dnβ1 average was 48.53% (n = 2 animals per genotype). In the cerebellum only 1 animal per genotype was analysed with wild type average of 48.77% and 50.0% for the dnβ1. A Student's t test was used for statistical analysis.

Following the same strategy used with the P9-P10 tissue, g-ratio was performed in different areas of the CNS (i.e., spinal cord, cerebellum and optic nerve). The morphology of myelin was unaltered in these regions with average g-ratios for control and mutants being 0.79 and 0.78 in spinal cord, 0.77 and 0.78 in cerebellum, and 0.79 ± 0.01 and 0.81 ± 0.02 in optic nerve, respectively (Fig. 3.11 – 3.13) . This demonstrated that there were no differences in terms of myelin thickness and compaction of myelin in these structures between mutant and wildtype animals. Originally, the project was focused only on areas of small diameter axons (i.e., optic nerve) and samples of the spinal cord and cerebellum were only collected from one pair of animals to check the quality of perfusion. These were later analysed, but only one sample per genotype was available. Therefore, no statistical test was used with these samples, and a simple average g-ratio comparison was used.



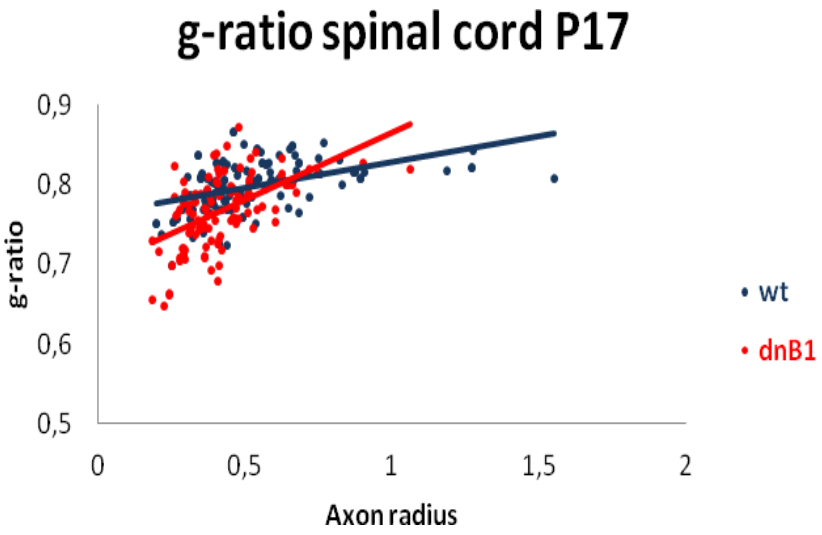


Figure 3.11 – Analysis of spinal cord of dominant negative β1 integrin mice at postnatal day 17. Upper panel: representative electron micrographs of spinal cord of wildtype and dominant negative β1 integrin mice at postnatal day 17. Magnification: 3.90K. Scale bar: 2 µm. Bottom panel: scatter plot displays g-ratios of individual myelinated axons as a function of their respective axon sizes (1 animal analysed per genotype). Blue circles represent wildtype (n = 153 axons; average g-ratio = 0.79) and red circles represent dominant negative β1 integrin mice (n = 112 axons; average g-ratio = 0.78).

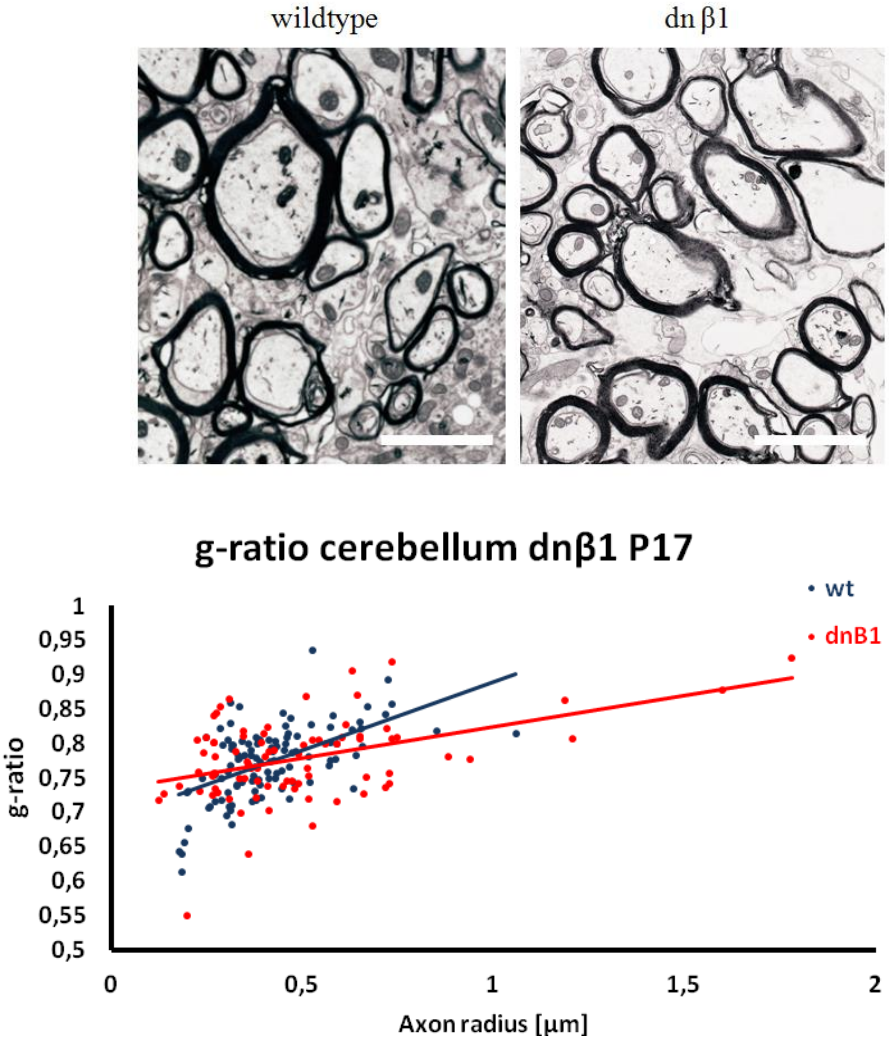


Figure 3.12 – Analysis of cerebellum of dominant negative β1 integrin mice at postnatal day 17. Upper panel: representative electron micrographs of cerebellum of wildtype and dominant negative β1 integrin mice at postnatal day 17. Magnification: 3.90K. Scale bar: 2 µm. Bottom panel: scatter plot displays g-ratios of individual myelinated axons as a function of their respective axon sizes (1 animal analysed per genotype). Blue circles represent wildtype (n = 107 axons; average g-ratio = 0.77) and red circles represent dominant negative β1 integrin mice (n = 83 axons; average g-ratio = 0.78).

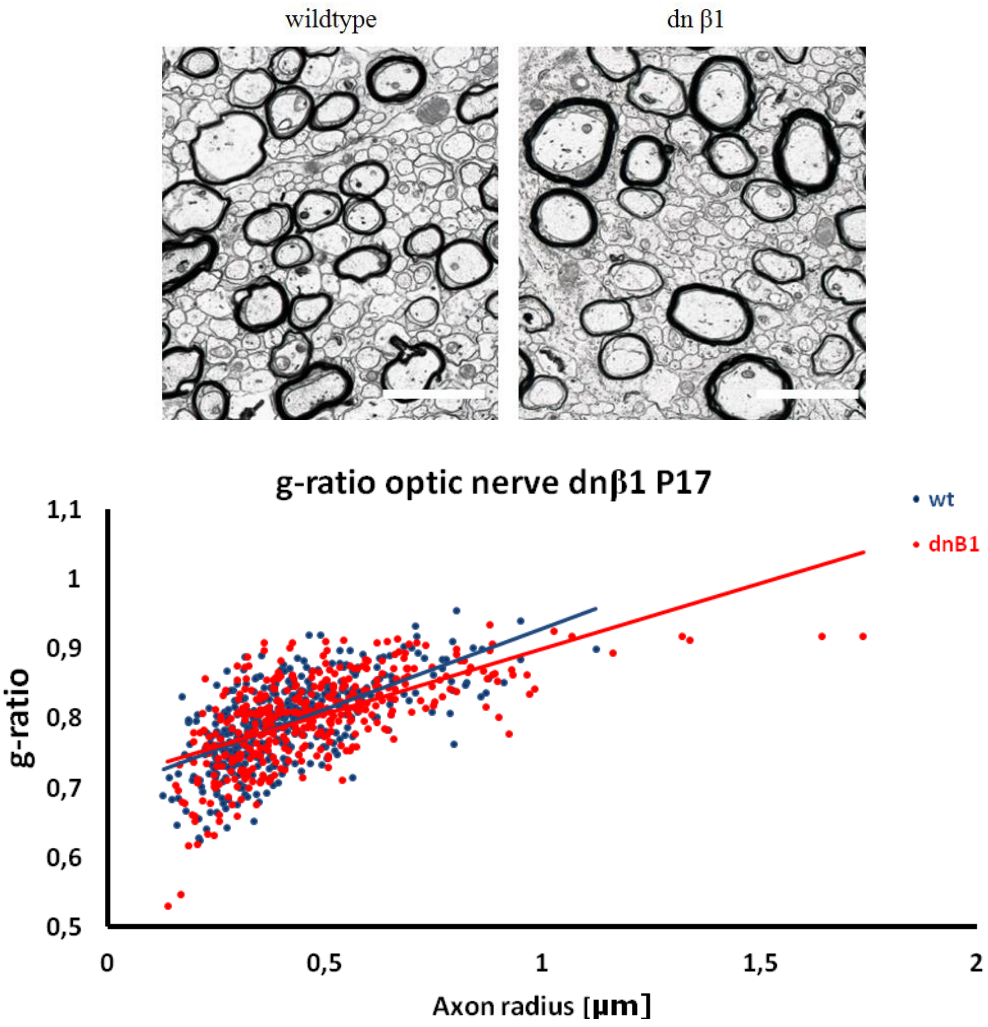


Figure 3.13 – Analysis of optic nerve of dominant negative β1 integrin mice at postnatal day 17. Upper panel: representative electron micrographs of spinal cord of wildtype and dominant negative β1 integrin mice at postnatal day 17. Magnification: 3.90K. Scale bar: 2 µm. Bottom panel: scatter plot displays g-ratios of individual myelinated axons as a function of their respective axon sizes, with the linear regression of the measurements for each animal (4 animals analysed per genotype). Blue circles represent wildtype (n = 401 axons; average g-ratio = 0.79 ± 0.01) and red circles represent dominant negative β1 integrin mice (n = 400 axons; average g-ratio = 0.81 ± 0.02). P = 0.65. Statistical analysis done by performing a Student's t test.

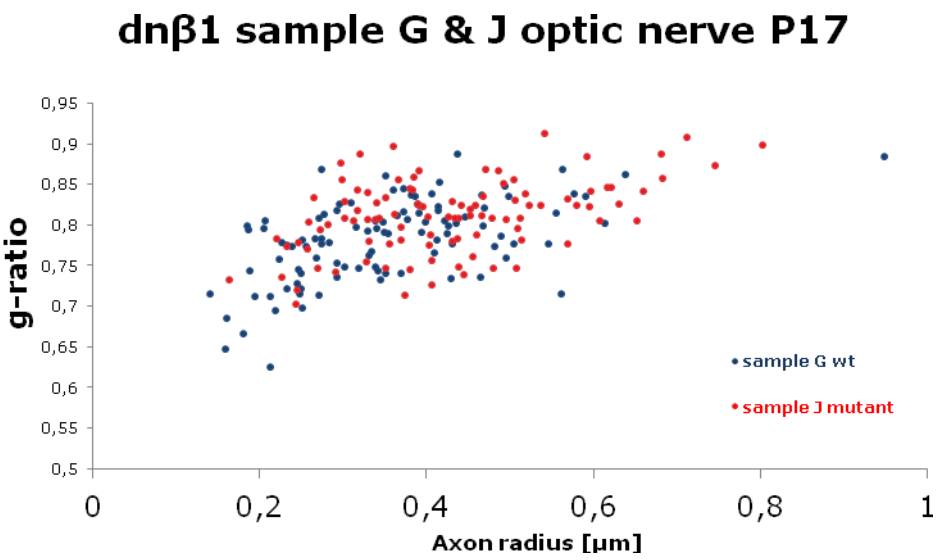


Figure 3.14 – Wildtype mice have a small population of myelinated small calibre axons which are absent from dominant negative β1 mice. This were the premilinary results where the dominant negative β1 integrin phenotype was first observed and based on this observation, a new method of myelin quantification (Percentage of myelination) was developed to further explore this phenotype. Scatter plot displays g-ratios of individual myelinated axons as a function of their respective axon sizes (1 animal analysed by genotype). Blue circles represent wildtype (n = 101 axons; average g-ratio = 0.78) and red circles represent dominant negative β1 integrin mice (n = 100 axons; average g-ratio = 0.81).

Interestingly, while analysing this data we noticed that there was a small population of myelinated axons of small calibre axons present in the wildtype, which was absent in the mutant mice (Fig. 3.14), suggesting that wildtype oligodendrocytes would be able to recognize smaller axons and initiate myelination while dnβ1 oligodendrocytes could not. Our hypothesis was that mutant oligodendrocytes required a higher threshold in terms of axon size to initiate myelination. In order to test our hypothesis we performed an analysis of the percentage of myelinated axons, where we compared the relationship between axon size and the percentage of myelination. The quantification of the percentage of myelination was done by measuring the axon diameter of at least 400 axons, and scoring them as myelinated or unmyelinated. The percentage of myelinated axons was plotted against 0.1µm intervals of the axon diameter. Spinal cord, cerebella and optic nerve were analysed, with spinal cord and

cerebella not showing a difference with an average percentage of myelination for spinal cord being 64.77 and 66.40 for wildtype and mutant mice, respectively; and in cerebella, 64.85 and 65.62 for wildtype and mutant mice, respectively (Fig. 3.15 and 3.16). According to the initial study plan, the focus of this project was on the optic nerve, therefore there were limited samples from both spinal cord and cerebellum, since these were only collected as a control on the quality of the perfusion. Therefore, when only one or two samples per genotype was available, it was not possible to statistically analyse the data, and an average comparison was performed.

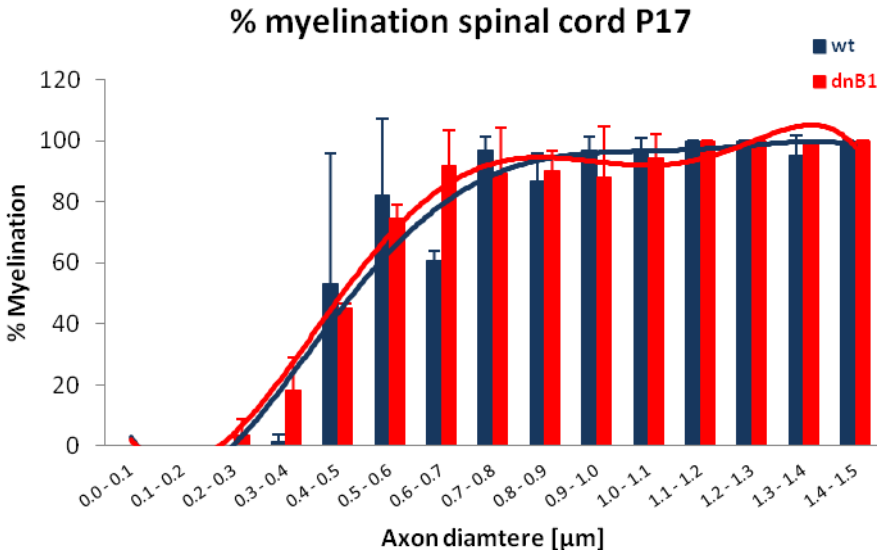


Figure 3.15 – Spinal cord of dominant negative β1 integrin shows no alteration of myelination at postnatal day 17 (P17). Graph representing 2 animals for each genotype of the percentage of myelinated axons with a polynomial trend line curve adjusted to the data shows no difference between the wildtype (average = 64.77, n=1021 axons) and dnβ1 (average = 66.40, n=735 axons). No statistical analysis was performed due to low number of samples, this graph shows the trend of the percentage of myelination in the spinal cord of dominant negative β1 mice.

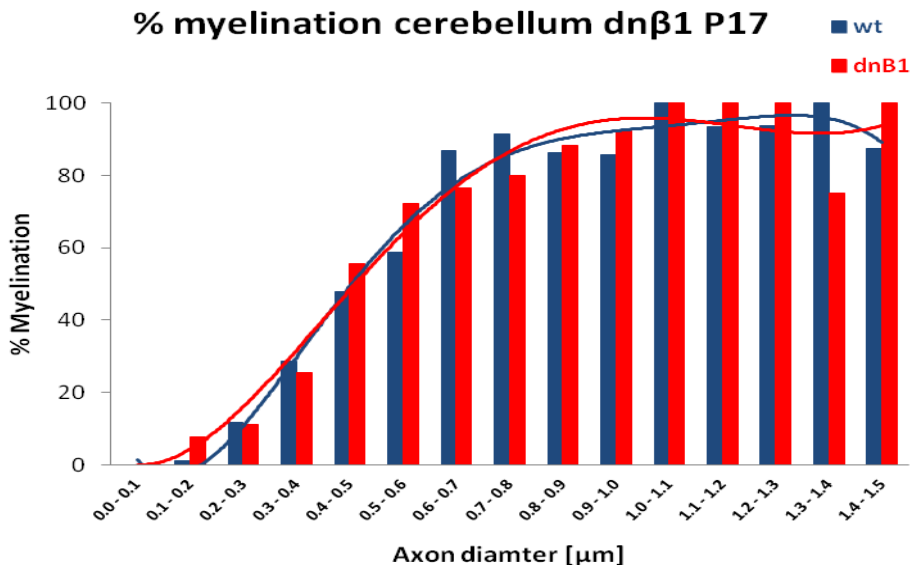


Figure 3.16 – Analysis of the cerebellum of dominant negative β1 integrin mice at postnatal day 17 (P17) shows no alteration of myelination. Graph representing 1 animal for each genotype of the percentage of myelinated axons with a polynomial trend line curve adjusted to the data shows no difference between the wildtype (average = 64.85, n = 61 axons) and dnβ1 (average = 65.62, n = 416 axons).

In the optic nerve, the percentage of myelination corroborated the observations seen with the g-ratios and confirmed that disruption of β1 integin signalling affects myelination with dnβ1 mice having a lower percentage of myelination in small diameter axons (Fig. 3.17).

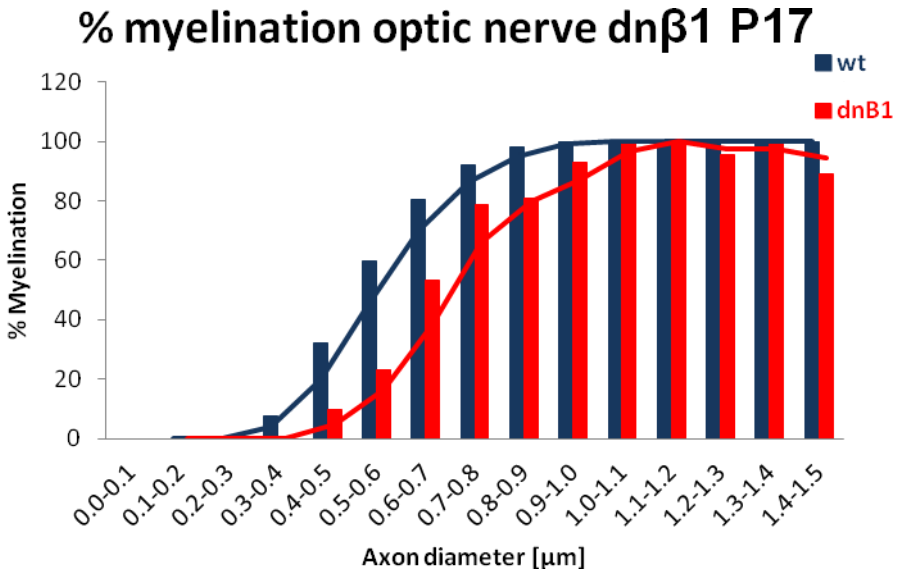


Figure 3.17 – The axon diameter required to initiate myelination is increased in the dominant negative β1 integrin mice. Representative graph from one litter showing the percentage of myelinated axons plotted against 0.1µm intervals of axon diameter depicts a reduced percentage of myelination in the dominant negative β1 integrin mice when compared to the wildtype (*, $P < 0.05$), with a more striking discrepancy in axons with diameter below 0.6 µm (**, $P< 0.01$). Wild type mice showed an average of percentage of myelination of 66.30 ± 10.09% (n = 2506 from 3 animals), contrasting to the dnβ1 average of 62.08 ± 10.55%(n = 2494 from 3 animals), with a ($P = 0.023$). When plotting the data to consider only small diameter axons (< 0.6 µm) the differences are more prominent ($P = 0.0085$). A two-way ANOVA was performed to statistically analyse the data.

The percentage of myelination showed that mutant mice need a higher axon diameter to initiate myelination ($P = 0.023$), with percentage of myelination being 66.30 ± 10.09%, for wildtype (n = 2506 from 3 animals) and 62.08 ± 10.55% for dnβ1 (n = 2494 from 3 animals). This difference is accentuated in small axons with a diameter of less than 0.6 µm ($P = 0.0085$) and it is more striking in the 0.3-0.4 µm interval ($P = 0.0023$).

To further assess the process of myelination in the dnβ1 mice, we carried out the same type of investigation at P28, an age where myelination is complete. Since this phenotype was restricted to the optic nerve we repeated the same ultrastructural

analysis in this area. The g-ratio of optic nerve showed that myelin integrity is preserved (P=0.57) in these mice with average g-ratios of 0.79 ± 0.013 (3 animals, n=300 axons) for wildtype and 0.77 ± 0.02 for mutant (3 animals, n=300 axons) (Fig. 3.18).

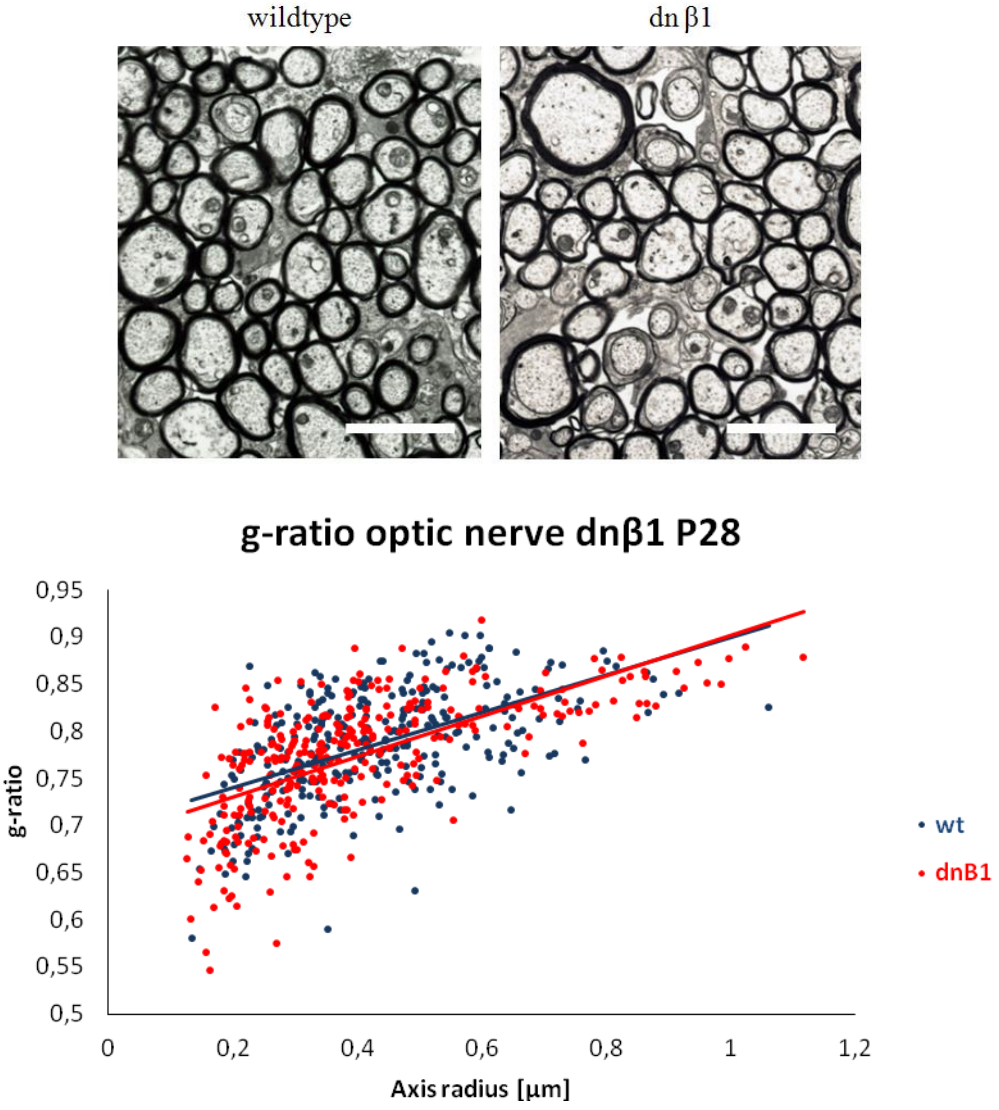


Figure 3.18 – Analysis of optic nerve of dominant negative β1 integrin mice at postnatal day 28. Upper panel: representative electron micrographs of optic nerve of wildtype and dominant negative β1 integrin mice at postnatal day 28. Magnification: 3.90K. Scale bar: 2 µm. Bottom panel: scatter plot displays g-ratios of individual myelinated axons as a function of their respective axon sizes, with the linear regression of the measurements for each animal (3 animals analysed per genotype). Blue circles represent wildtype (n = 300 axons; average g-ratio = 0.79 ± 0.013) and red circles represent dominant negative β1

integrin mice (n = 300 axons; average g-ratio = 0.77 ± 0.02). P = 0.57. Statistical analysis performed by a Student's t test.

We further investigated if at this later stage of myelination the percentage of myelination would still be affected at P28. We observed no differences (P=0.97) between the wildtype littermates (73.46 ± 10.40, n=2421 axons from 3 animals) and their mutant counterparts (75.31 ± 10.28, n=2750 axons from 3 animals), showing that our phenotype of delayed myelination is transient and β1 integrin signalling is only necessary for the initiation of myelination, and is not a long-lasting effect throughout the entire process of myelination (Fig. 3.19). To further assess any possible alteration in terms of myelination and axon calibre, we have plotted the percentage of myelinated axons according to small (≤ 0.6 μm) and large (> 0.6 μm) diameter (Fig. 3.19). This demonstrated that at P28, both wildtype and dnβ1 mice have the same percentage of myelinated axons, showing that mutant oligodendrocytes are able to restore their ability of myelinating small diameter axons with time. When comparing the percentage of myelinated axons in P17 and P28 in different axon diameter intervals we observed that for axon diameters of 0.2-0.3 μm, the percentage of myelination is 1.2% at P17 and 6.1% at P28; for axon diameters of 0.3 – 0.4 μm, percentages of myelination are 12.7% and 51.4% at P17 and P28, respectively; for axon diameters of 0.4 – 0.5 μm, the percentage of myelination is 37.2% at P17 versus 83.6% at P28. The increase observed from P17 to P28 shows that mutant oligodendrocytes restore their ability to myelinate small calibre axons over time.

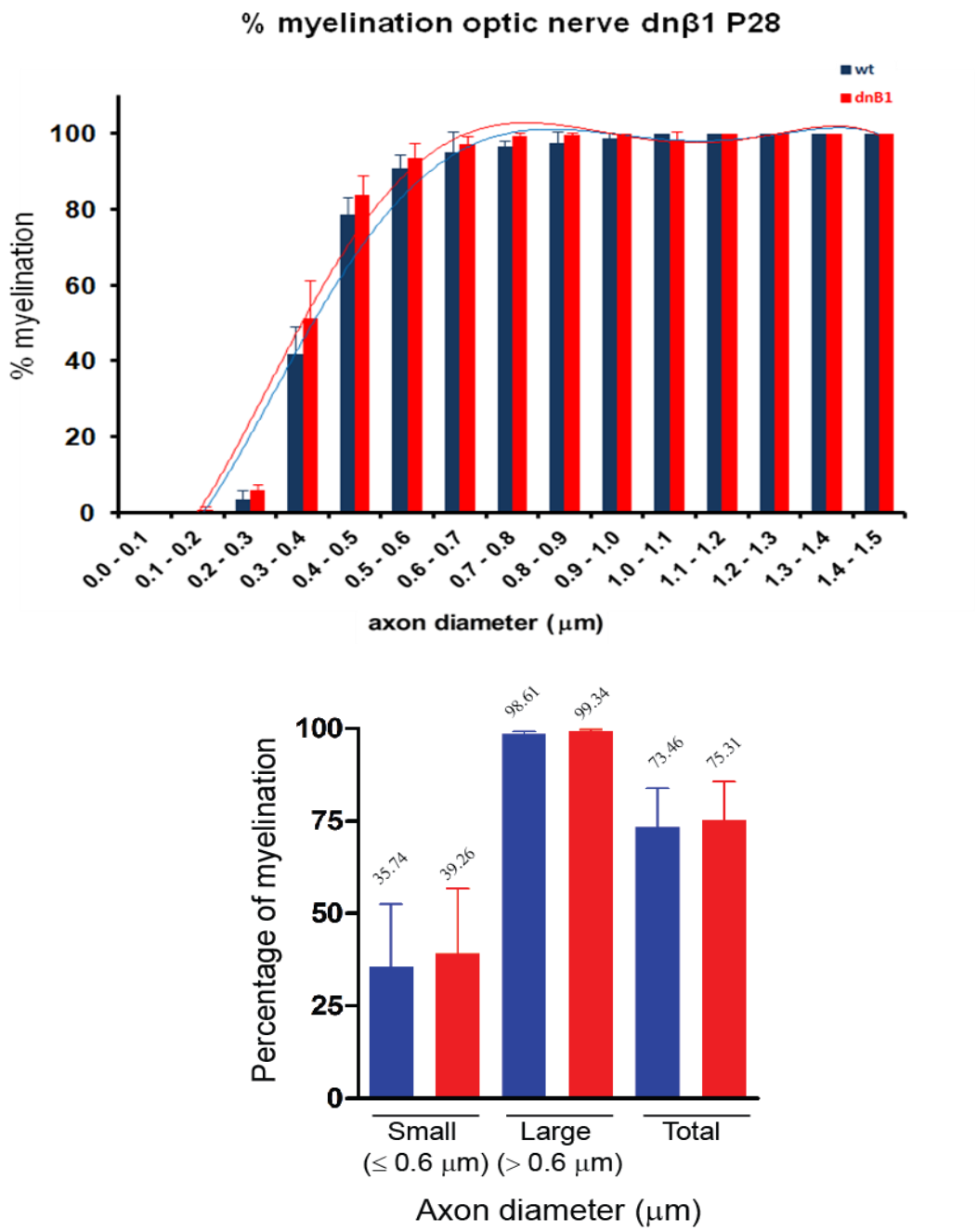


Figure 3.19– The threshold diameter needed for initiation of myelination in the dominant negative β1 mice is restricted to postnatal day 28 (P28). Upper panel: Percentage of myelinated axons in dominant negative β1 mice at postnatal day 28 (P28) (average of 75.31 ± 10.28, n = 2750 axons from 3 animals) shows no difference when compared to wildtype mice (average of 73.46 ± 10.40, n = 2421 axons from 3 animals) (P = 0.97), showing that disruption of β1 integrin signaling affects predominantly the initial stages of myelination. Bottom panel: The percentage of myelination in the mutant does not significantly differ from wild-type in small, large or total axons (same n numbers as upper panel). A Student's t test was used to statistically analyse the data.

To further access the role of β1 integrin in myelination, β1 integrin blocking antibody was added to myelinating cocultures of dorsal root ganglia (DRGs) where oligodendrocyte precursor cell (OPCs) were added. Cocultures were supplemented with blocking antibody at different time points: 0, blocking antibody was added at the same time as OPCs were seeded onto DRGs; 1, one day after adding OPCs; and 3, three days after OPCs were added to DRGs. These results support the idea that β1 regulates oligodendrocyte myelination since it was observed a decreased in the percentage of myelinating oligodendrocytes (calculated by dividing the number of oligodendrocytes with established internodes for the number of MBP positive oligodendrocytes) in all time points when compared with controls (no antibody and λ1 Monoclonal Immunoglobulin Isotype Standard antibody).

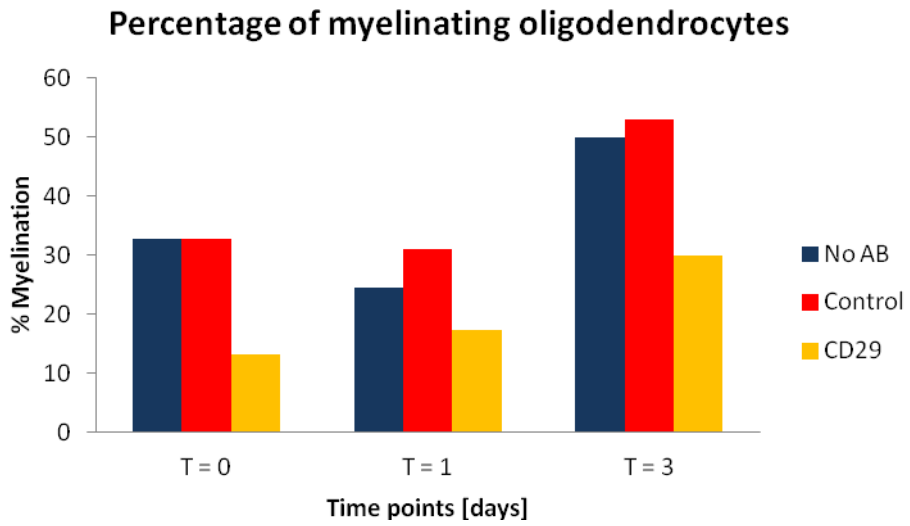


Figure 3.20 – β1 integrin reduces myelination in vitro. β1 integrin blocking antibody was added to myelinating cocultures and reduced myelination in different time point. This graph comprises data from one set of experiments. Each bar represents 3 cover slips. Twelve rat embryos were dissected to extract dorsal root ganglia (DRGs for neuronal culture) and a further 16 rat pups were used to extract oligodendrocytes. Since it is only one set of experiments no statistical test was used.

3.2.2 Analysis of the dominant negative β3 integrin mice

Dominant negative β3 integrin (dnβ3) mice were also engineered by Dr. Joana Câmara following the same strategy described in the previous section (Câmara *et al.*, 2009). These mice were used as a control since β3 integrin is not expressed in myelinating oligodendrocytes and therefore any phenotype observed in the dnβ1 would be specific to β1 integrin and not an artefact of the construct/transgenic-approach. As previously observed in the dnβ1 mutant, dnβ3 mice show that the trend in terms of the percentage of unmyelinated axons is not different, with averages of 43.66 % (n = 1486 axons from three animals) and 37.91 (n = 1140 axons from 2 animals) for wildtype and mutant, respectively (Fig. 3.21).

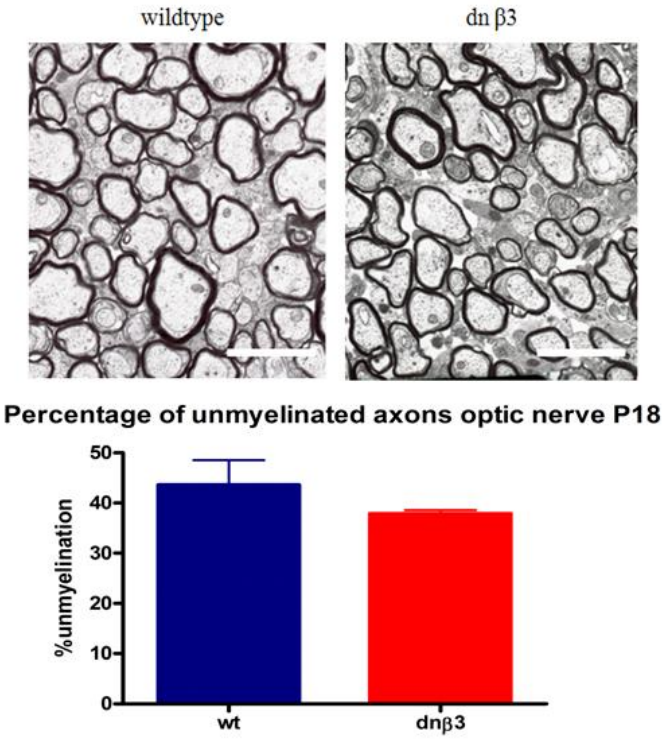


Figure 3.21 – Normal myelination of optic nerve of dominant negative β3 integrin mice (P18). Upper panel: representative electron micrographs of wildtype and dnβ3 mice optic nerve at postnatal day 18. Magnification: 3.90K. Scale bar: 2 μm. Bottom panel: The bars represent the mean of the percentage of unmyelinated axons. Blue bars represent wildtype animals (average of 43.66, from 3 animals, n = 1486 axons). Red bars represent dnβ3 animals. (average of 37.91, 2 animals, n = 1140 axons).

The dnβ3 line was a helpful control for the dnβ1 line since it shares the same construct which includes the cytoplasmic β integrin subunit. The percentage of myelination of these mice allowed us to exclude that a phenotypic change in myelination resulted from the transgenic approach, with no significant difference when compared to wildtype animals (P = 0.24) when looking at average percentage of myelination (wildtype: 54.07 ± 10.41 (n = 1397 axons from 3 animals, dnβ3: 58.73 ± 10.54 (n = 1416 from 3 animals)) (Fig. 3.22). This proves that the delay in myelination observed in the dnβ1 is due to β1 integrin signalling rather than from a nonspecific effect of transgenic expression.

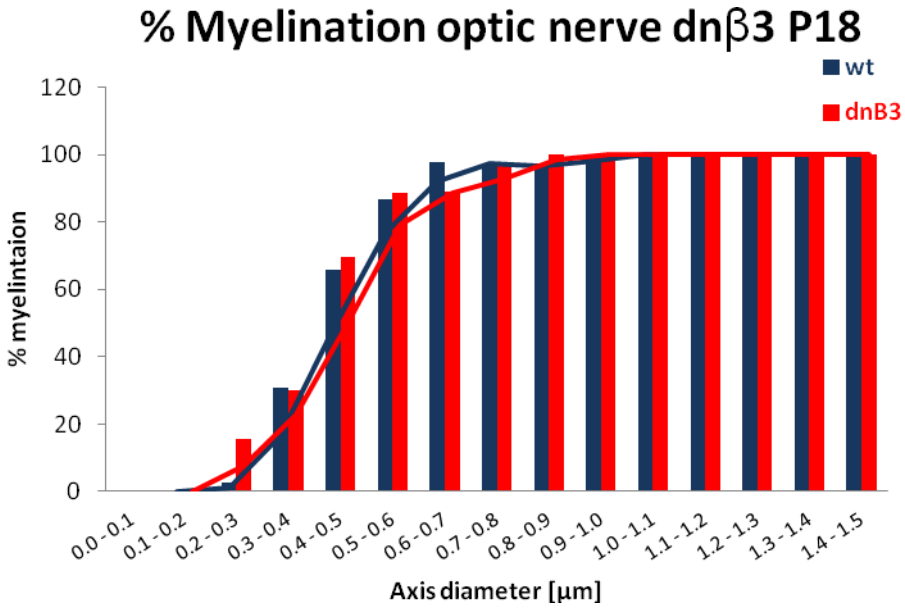


Figure 3.22 – Dominant negative β3 mice show no delay of myelination. Representative graph of one litter of percentage of myelinated axons plotted against 0.1 µm intervals, showing no difference (P = 0.24) in terms of the threshold necessary for myelination between wildtype (average of 54.07 ± 10.41, n = 1397 axons from 3 animals) and dnβ3 mice (average of 58.73 ± 10.54, n =1 416 from 3 animals). Statistical analysis performed by two-way ANOVA.

As predicted, the delay in myelination was specific to β1 integrin signalling and not due to the transgenic approach taken. To further prove this point we have repeated the percentage of myelination in the optic nerve of dnβ3 mice at P28 and no

difference was detected with average percentage of myelination being 69.75 (n = 502 axons from 1 animal) for the wildtype and 72.31 (n = 364 axons from 1 animal) in the dnβ3 mice (Fig. 3.23).

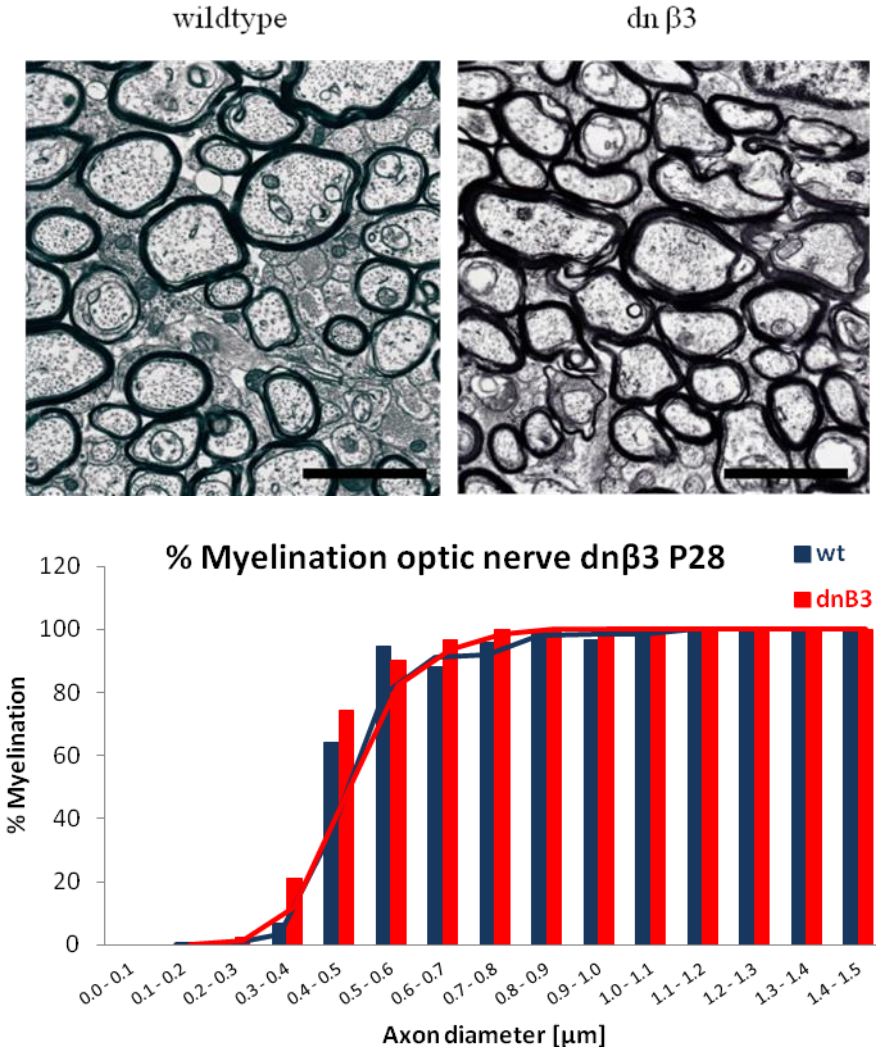


Figure 3.23 – Dominant negative β3 integrin mice show no abnormalities in myelination. Upper panel: representative electron micrographs of wildtype and dnβ3 mice optic nerve at postnatal day 28. Magnification: 3.90K. Scale bar: 2 µm. Bottom panel: representative graph of one litter of percentage of myelinated axons plotted against 0.1 µm intervals, showing a similar myelination between the dnβ3 (average of 72.31, n = 364 axons from 1 animal) and wildtype mice (average of 69.75, n = 502 axons from 1 animal).

3.2.3 Analysis of the β1 integrin knockout mice

The β1 integrin knockout (β1KO) mice were kindly supplied by Dr. João Relvas and an extensive protocol for the transgenic approach has been previously described (Benninger *et al.*, 2006). Benninger and co-workers concluded that by conditional inactivation of the β1 integrin gene in CNP-expressing oligodendrocytes, β1 integrin was not required for CNS axon ensheathment, myelination and remyelination (Benninger *et al.*, 2006). It was proposed that due to the early expression of CNP, these oligodendrocytes would be deprived of β1 integrin expression from an early age (premyelinating stage) and that compensatory mechanisms (perhaps from another integrin) would take place and therefore inhibit a phenotype in these mice. We received 3 samples from a wildtype genotype and 3 from the β1 integrin knockout (these were littermates) and proceeded with the same type of analysis applied to the previous transgenic animals. These animals showed no difference in terms of percentage of unmyelinated axons with an average of 66.72 ± 8.98 (n = 2292 axons) for wildtype, and 62.96 ± 6.28 (n = 2231 axons) for β1 KO (Fig. 3.24).

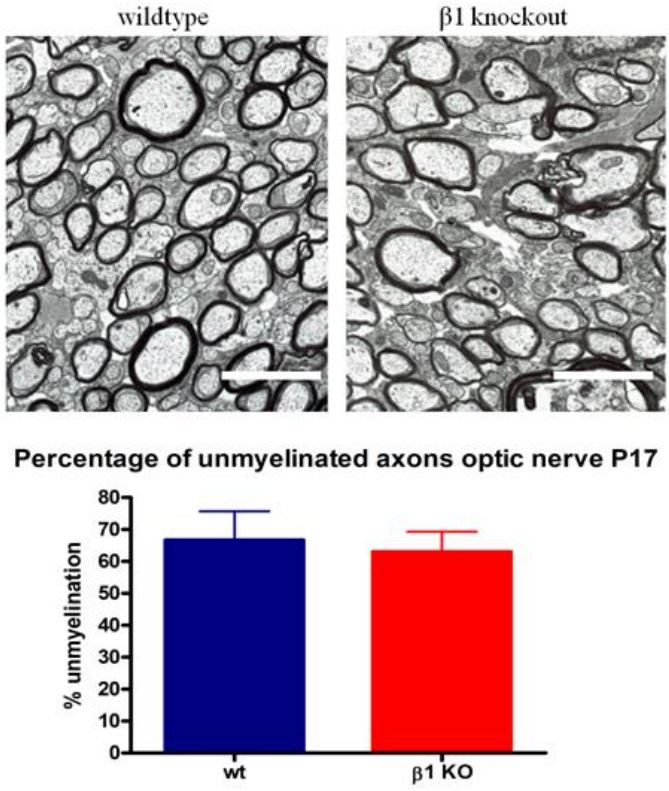


Figure 3.24 – Percentage of unmyelinated axons of optic nerve of β1 integrin knockout mice (P17). Upper panel: representative electron micrographs of optic nerve of wildtype and β1 integrin knockout mice at postnatal day 17. Magnification: 3.90K. Scale bar: 2 μm. Bottom panel: Percentage of unmyelinated axons of the optic nerve of wildtype and β1 integrin knockout mice at postnatal day 17.The bars represent the average of the percentage of unmyelinated axons. Blue bars represent wildtype animals. Red bars represent β1 KO animals. Optic nerve bars represent 3 animals, wildtype average = 66.72 ± 8.98 (n = 2292 axons) and β1 KO average = 62.96 ± 6.28 (n = 2231 axons), *P*=0.75. A Student's t test was executed for statistical analysis.

Performing the same analysis of the percentage of myelination in these mice showed no phenotype (Fig. 3.25), consistent with previous findings. Compensation by other integrins has been previously reported in *Drosophila* embryogenesis. In *Drosophila* PS integrins are necessary for the correct migration pattern during embryogenesis. Absence of this integrin results in disorganization of the visceral mesoderm and a migration delay. In contrast, ablation of either PS1 or PS3 (integrins from the PS integrins family) causes a modest delay in migration, or no effect, respectively (Martin-Bermudo *et al.*, 1999).

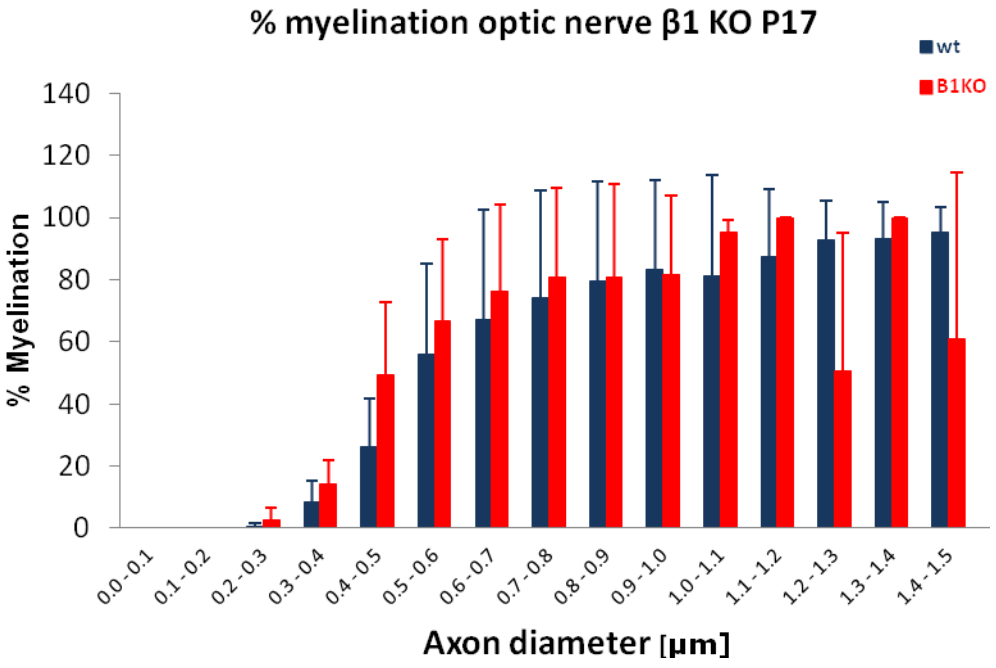


Figure 3.25 – Percentage of myelination of optic nerve of β1 integrin knockout mice at postnatal day 17. As expected from previous studies in these mice, no alterations in myelination were observed, giving rise to speculation that a knockout of β1 integrin at an

early stage will lead to compensatory mechanisms that ensure a correct and appropriate myelination. The bars represent the mean of the percentage of myelinated axons categorized by axon diameter. Blue bars represent wildtype animals. Red bars represent β1 KO animals. Average percentage of myelination for wildtype mice = 56.41 ± 9.79 (n = 2292 axons, 3 animals), and for β1 KO mice = 57.30 ± 9.45 (n = 2231 axons, 3 animals). P = 0.67. A two-way ANOVA was performed for statistical analysis.

3.2.4 Analysis of the focal adhesion kinase knockout mice

Focal adhesion kinase (FAK) is a downstream protein of β1 integrin and an integral part of integrin signalling. FAK is activated after binding to the integrin cytoplasmic domain with subsequent phosphorylation. It has been previously reported that loss of FAK results in hypomyelination of the optic nerve *in vivo* (Forrest *et al.*, 2009). We reasoned that if the phenotype seen in the dnβ1 mice was due to disturbances of the β1 integrin signalling then transgenic mice for downstream molecules of the same pathway would show the same delay in myelination. To test our hypothesis, we analysed focal adhesion kinase knockout mice (FAK KO), provided by Professor Peter Brophy and described previously (Grove *et al.*, 2007). Briefly, these mice have ablated FAK expression achieved by crossing mice homozygous for a floxed FAK allele with a Cnp-Cre line (Grove *et al.*, 2007). Initially, we investigated the integrity of myelin in these mice by analysing the g-ratio in P16 mice. As previously carried out, we initiated the analysis of the FAK KO mice by quantifying the percentage of unmyelinated axons, which in the wildtype was 66.46% (n = 1888, from two animals) and in the mutant was 76.65% (n = 1542 axon, from two animals), showing that the trend of unlyelinated axons does not differ from both genotypes (Fig. 3.26, middle panel). The myelin integrity of these mice is normal, with 3 wildtype animals and an average g-ratio of 0.78 (n = 300 axons) and the 2 mutant animals with and average g-ratio of 0.80 (n = 200 axons) (Fig 3.26, bottom panel).

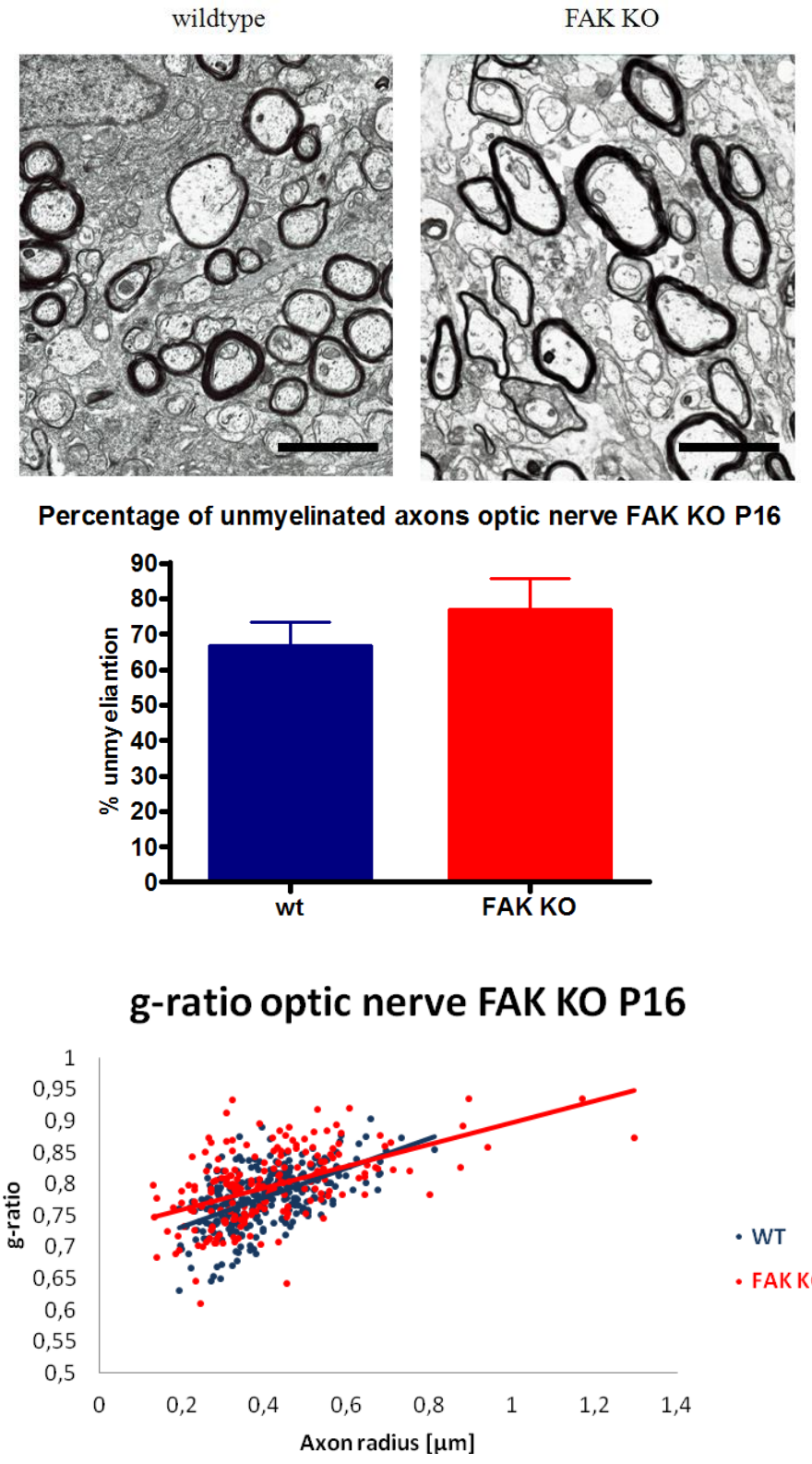


Figure 3.26 – Normal myelination and myelin thickness in the focal adhesion kinase knockout mice in the optic nerve at postnatal day 16 (P16). Upper panel: representative electron micrographs of optic nerve of wildtype and FAK knockout mice at postnatal day 16.

Magnification: 3.90K. Scale bar: 2 µm. Middle panel: Percentage of unmyelinated axons of the optic nerve of wildtype and FAK knockout mice at postnatal day 16.The bars represent the average of the percentage of unmyelinated axons. Blue bars represent wildtype animals. Red bars represent FAK KO animals. Optic nerve bars represent 2 animals, wildtype average = 66.46 (n = 1888 axons) and FAK KO average = 76.65 (n = 1542),. Bottom panel: scatter plot displays g-ratios of individual myelinated axons as a function of their respective axon sizes, with the linear regression of the measurements for each animal. Blue circles represent wildtype (n = 300 axons, from 3 animals; average g-ratio = 0.78) and red circles represent FAK KO mice (n = 200 axons, from 2 animals; average g-ratio = 0.80). .

These initial results corroborate the findings in the dnβ1 animals, where these mice show no defect in the thickness of myelin or oligodendrocyte ability to compact myelin. To test our hypothesis that FAK KO mice share the same delay in myelination seen in the dnβ1, we analysed the percentage of myelination and saw a significant reduction in myelination (P = 0.087) with averages of 64.93 ± 10.70% (n = 2560 axons, from 4 animals) for wildtype and 57.37 ± 10.38% for mutants (n = 2186 axons, from 4 animals) (Fig. 3.27).

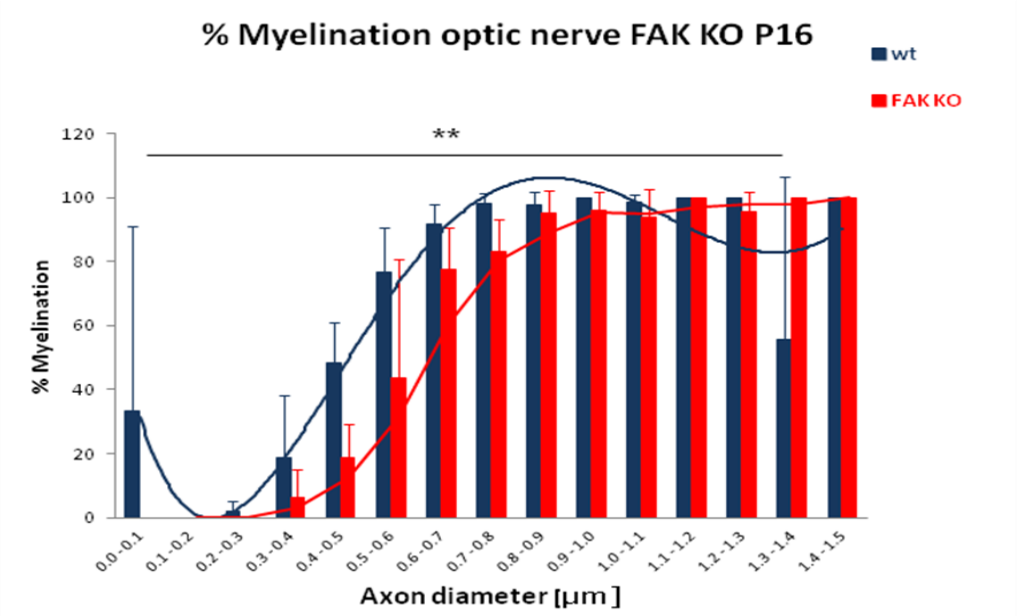


Figure 3.27 – Increased threshold for myelination in the focal adhesion kinase knockout mice. Graph shows the percentage of myelinated axons in the optic nerve of focal adhesion kinase knockout (FAK KO) mice and their wildtype counterparts with respect to the axon diameter. A polynomial trend line was adjusted to the data. FAK KO (average of 57.37 ± 10.38%, n = 2186 axons, from 4 animals) show a shift of the dose-response curve to the

right that is significant from the wildtype (average of 64.93 ± 10.70%, n = 2560 axons, from 4 animals) (**, P<0.001). A two-way ANOVA was performed for statistical analysis.

While analysing these mice, it was clear, especially from one particular litter that these animals presented several myelin aberrations (such as "loops" of uncompacted myelin (Fig. 3.28 A, B) and "double wraps" where more than one axon is wrapped in the same myelin sheath (Fig 3.28 C, D). A significant difference (P = 0.0069) was observed in the P16 litter with average percentage of myelin aberrations of 1.62 ± 0.28% and 5.19 ± 0.11% for wildtype and mutant, respectively. At P21, data trend seemed to be the same with percentage of myelin aberrations with wildtype presenting an average of 0.98 and FAK knockouts having an average of 0.96 (Fig. 3.28). This might evidence the transient aspect of the phenotype described, with oligodendrocytes eventually recovering their ability to myelinate smaller axons and correctly compact myelin.

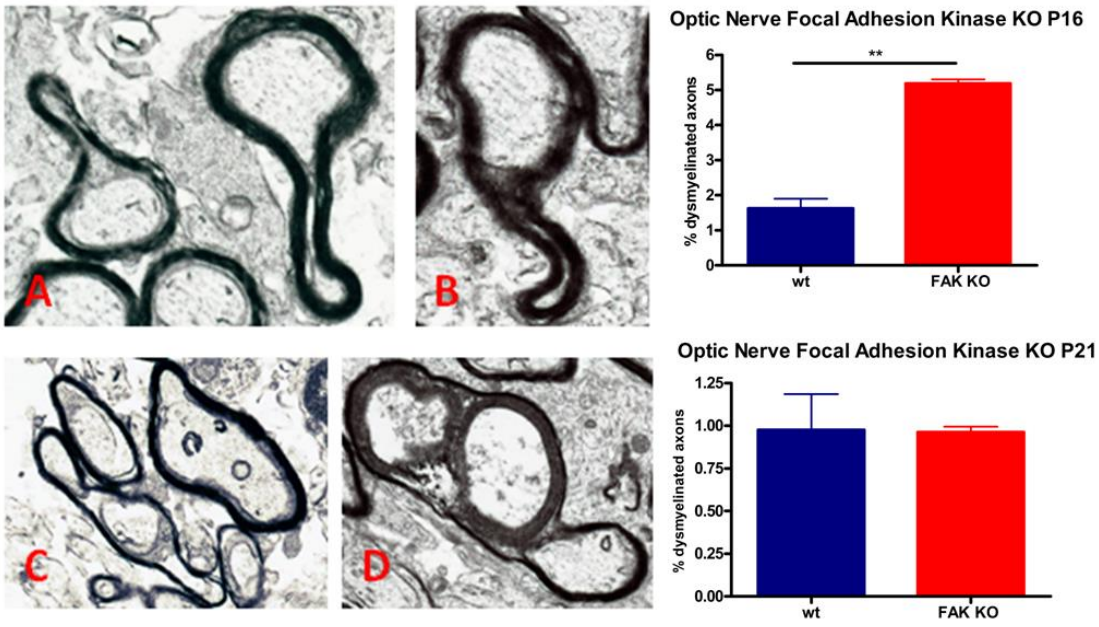


Figure 3.28 –Percentage of myelin aberrations in the conditional focal adhesion kinase knock out mice at postnatal day 16 and 21 (P16 and P21). The P16 litter, comprised of 4 wildtype samples and 4 knockouts, and the P21 litter was composed of 6 wildtype samples and 2 knockouts. The graph represents the average of the percentage of myelin aberrations of each sample, in the wildtype the P16 litter had 1,60% myelination, while in the P21 litter wildtype had 0,98% myelination. In the knockouts, at P16 there was 5,19% myelination in contrast to 0,96% myelination in the P21 litter. Myelin aberrations: **(A)**

and **(B),** Loops of myelin, **(C)** and **(D)** "double wraps". (**, P < 0.001). This analysis was performed by two-way ANOVA.

After showing that the FAK KO mice share the same phenotypic features as the dnβ1 mice, we analyzed adult mice to assess if the delay in myelination was also transient. We performed g-ratio analysis and calculated the percentage of myelination in P60 mice. G-ratios showed that at this age, the thickness of the myelin sheath is normal (P = 0.67) in the optic nerve with average g-ratios for wildtype of 0.83 ± 0.02 and 0.82 ± 0.02 for FAK KO mice (Fig. 3.29).

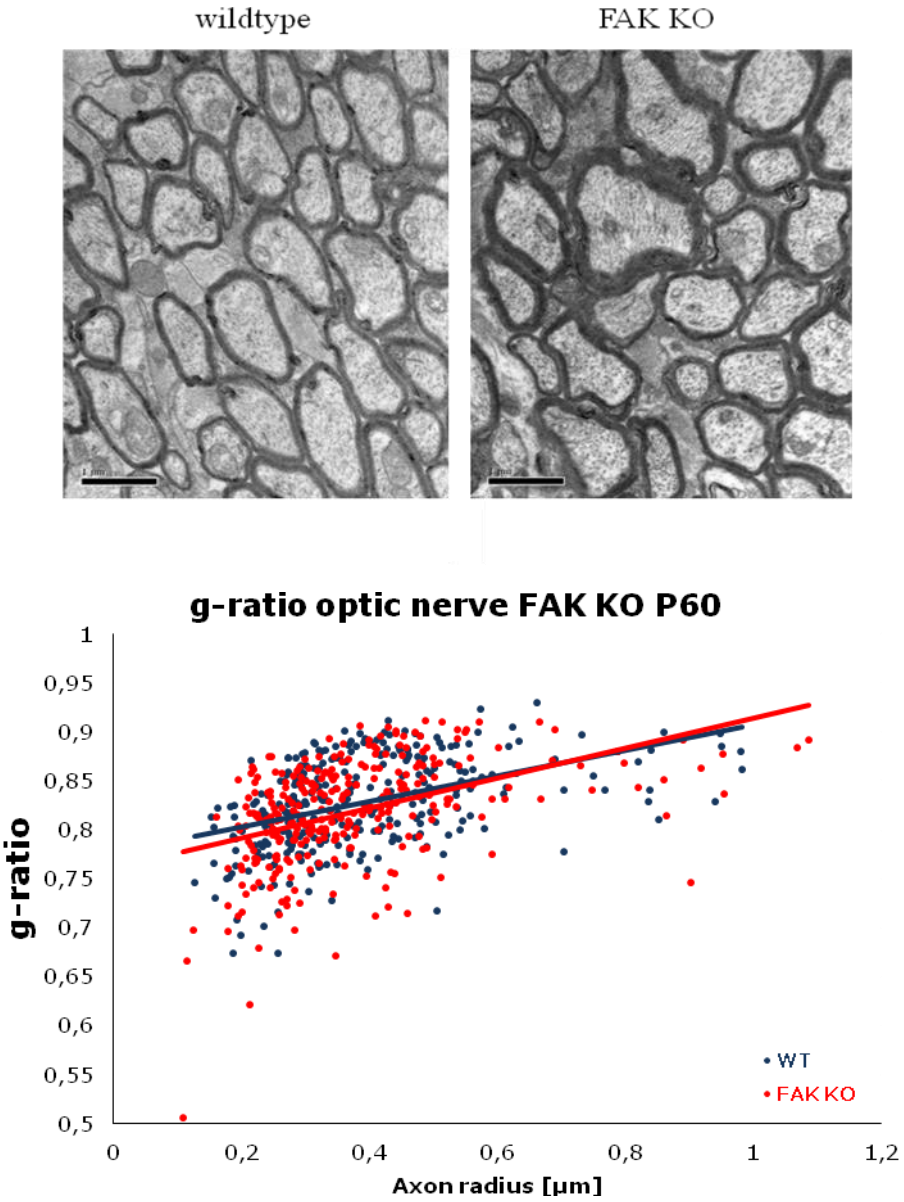


Figure 3.29– Normal myelination in the focal adhesion kinase knockout mice at postnatal day 60 (P60). Upper panel: Upper panel: representative electron micrographs of optic nerve of wildtype and FAK knockout mice at postnatal day 60. Magnification: 3.90K. Scale bar: 1 µm. Bottom panel: No significant differences (P = 0.67) in myelin thickness were detected in the optic nerve in the absence of FAK at P60 in the three animals analyzed for each genotype, with average g-ratios for wildtype and FAK KO mice of 0.83 ± 0.02 (n = 300 axons, from 3 animals) and 0.82 ± 0.02 (n = 300 axons, from 3 animals), respectively. A Student's t test was used for statistical analysis.

Similarly to what was observed in the dnβ1 mice, FAK KO mice also showed a transient effect, with P60 mice not showing a delay in myelination specific to small diameter axons. There is no difference (P = 0.78) between the percentage of myelinated axons in the wildtype 77.52 ± 10.09 (n = 1288, from 3 animals) when compared to mutant mice 74.40 ± 10.35 (n = 1268, from 3 animals) (Fig. 3.30). When we plotted the percentage of myelinated axons by small (≤ 0.6 µm) and large (> 0.6 µm) diameters, these values are comparable between genotypes (Fig. 3.30).

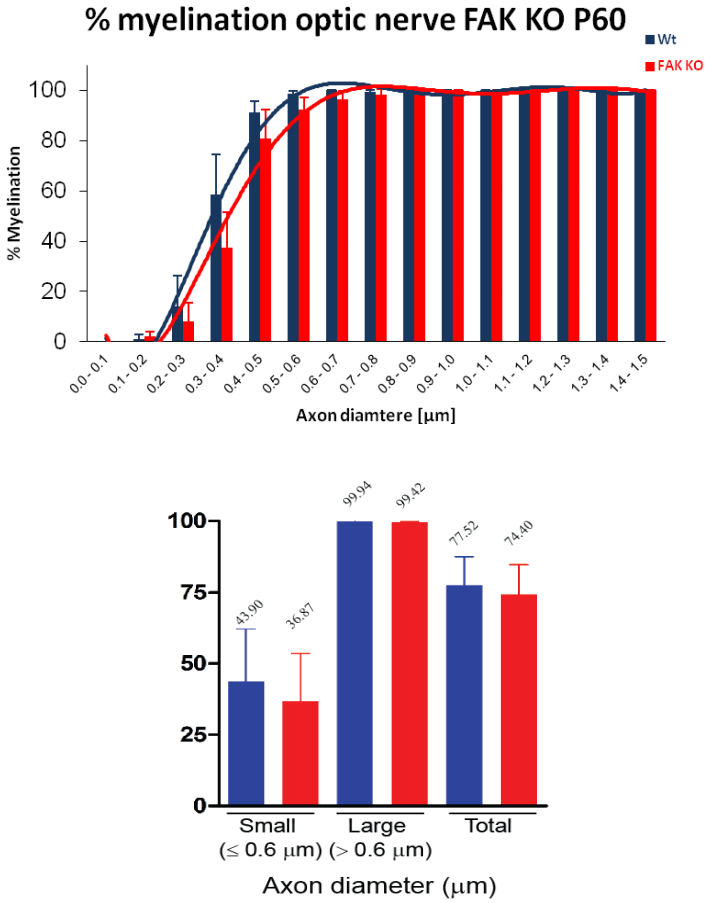


Figure 3.30 - Transient effect in myelination is observed in focal adhesion kinase knockout mice. Upper panel: Graph plotting the percentage of myelinated axons against the axon diameter at 0.1 µm intervals for P60 wild-type and FAK KO mice. A polynomial trend line was adjusted to the data. At P60, the percentage of myelinated for the FAK KO optic nerves (74.40 ± 10.35, n = 1268, from 3 animals) was not significantly different (P=0.78) from wild-type littermates (77.52 ± 10.09, n = 1288 from 3 animals). Bottom panel: Percentage of myelinated axons in the FAK KO mice (P60). As seen in the dnβ1 mice, the percentage of myelination in the mutant does not significantly differ from wild-type in small, large or total axons. A two-way ANOVA was performed for statistical analysis.

3.3 Discussion

β1 integrin has been implicated in central nervous system (CNS) myelination *in vitro*, but these findings have been difficult to translate into *in vivo* systems as many integrin knockouts are embryonic or prenatally lethal. In order to address β1 integrin myelination *in vivo*, our lab adopted a new transgenic approach by developing mice with dominant negative β1 integrin (dnβ1) expressed under the myelin basic protein (MBP) promoter that drives specific expression on myelinating oligodendrocytes. The dominant negative strategy used allowed us to analyse the effects of β1 integrin specifically in oligodendrocytes. The dnβ1 integrin transgenic mice bear a construct that comprises the cytoplasmic domain of the β1 integrin coupled to the extracellular and transmembrane domains of a small non-signalling α subunit of the human interleukin-2 receptor, which reduces signalling independently of ligand binding. After full characterization of β1 integrin expression of these mice, a structural analysis of the myelin was performed at different time points: postnatal day 9 (P9), when myelination is initiating; P17, when myelination is well underway but is not yet complete; and P28, when myelination is complete.

The number of myelin wraps, and consequently after compaction, the myelin thickness, has long been known to be proportional to the axon diameter, allowing for calculation of the g-ratio (Friede, 1972). Structural analysis of myelination by electron microscopy (EM) enabled us to quantify the g-ratio in dnβ1 mice. At P9, dnβ1 mice showed no impairment in myelination. Interestingly, in the optic nerve, a

small population of myelinated axons of small calibre which were present in wildtype mice were absent in the dnβ1 mice. In order to confirm these results, we generated a new method of analysing myelination by quantifying the percentage of myelinated axons binned by axon diameter, allowing for the comparison of major differences between genotypes in different categories of axons. Oligodendrocytes of dnβ1 mice myelinated small-diameter axons less efficiently than wildtype and control (dominant negative β3 integrin mice) in the optic nerve, demonstrated by a shift to the right of the dose-response curve created based on the percentage of myelinated axons. This delay in myelination was observed at P17, when myelination is not completed. Analysis of spinal cord and cerebellum showed that this phenotype was specific to the optic nerve, an area characterized by small diameter axons. Analysis of older mice showed that this is a transient effect and that oligodendrocytes eventually catch up and are able to myelinate the smaller axons.

As a control for the transgenic approach we also characterized dominant negative β3 integrin (dnβ3) mice. These mice were generated using the same transgenic method having their extracellular and transmembrane domains replaced by a small non-signalling α subunit of the human interleukin-2 receptor. β3 integrin is not normally expressed in myelinating oligodendrocytes, proving that the phenotype observed in the dnβ1 mice is specific to the β1 integrin and not a construct artefact. These mice showed no structural or myelinating abnormalities, nor the delay in myelination observed in the dnβ1 animals.

Further to the dnβ3 control, another two lines of mice were analysed, the focal adhesion kinase knockout (FAK KO) and the β1 integrin knockout (β1 KO). FAK is a downstream signalling molecule of the β1 integrin, and the FAK KO showed a similar effect than the one observed in the dnβ1 mice. The β1 KO showed no effect, consistent with the current literature, and may be explained by β1 integrin being deleted in oligodendrocytes at premyelinating stages, thereby likely allowing compensation by other integrins.

Together, these results showed no abnormalities in terms of myelin thickness, which suggests that dnβ1 oligodendrocytes, once they establish contact and myelinate an axon, do so according to its diameter. *In vitro* studies performed by Dr. Zhen Wang give more emphasis to the results described since internodal length of myelinated axons in co-culture with dominant-negative oligodendrocytes is normal (Câmara *et al.,* 2009). Since the delay in myelination in the dnβ1 is a transient effect, we suggest that these dnβ1 oligodendrocytes require a higher axon size threshold to start myelinating. To further support this view, this observation was specific to the optic nerve, a region where the population of fibres is homogenously small, and no differences were detected in spinal cord or cerebellum, areas characterized by large calibre axons. Together these results suggest that β1 integrin is required to detect a signal on the axon important to switch on the myelination program.

Oligodendrocytes with reduced β1 integrin signalling myelinate fewer small-diameter axons, leading to a delay in initiation of myelination without affecting the signalling pathways that control the formation of the myelin sheaths. Therefore, integrins contribute to the axoglial interactions that initiate myelination, but do not affect myelin thickness once axoglial interactions have been established.

Two different studies of the function of β1 integrin in central myelination appear contradictory (Benninger *et al.,* 2006; Lee *et al.,* 2006). Our project reconciles these studies. Benninger and co-workers reported the absence of a myelination phenotype in the CNS in a CNP-Cre β1 KO (the same mice analysed in this study, β1 KO) which corroborates our results that β1 integrin is necessary for initial axoglial contact but has no effect on myelin wrapping. Lee and co-workers generated dominant negative β1 integrin mice using a different strategy, where mice lack the C-terminal cytoplasmic tail (β1ΔC) in oligodendrocytes. These mice showed a hypomyelination of the CNS that might be explained by the transgenic approach. β1ΔC comprises the extracellular and transmembrane domains of the β1ΔC and lacks its intracellular domain, which is the reverse strategy taken with our dnβ1. As opposed to our mice where signalling is reduced independent of ligand binding (dnβ1 is unable to bind α subunits and thus to bind laminin), the β1ΔC mice are able to bind to β1 integrin

ligands, such as laminin, and heterodimerise with the α subunit, although due to the lack of the β tail, the signal is unable to be transmitted into the cell. If we consider β1 integrin as the main laminin receptor involved in CNS myelination, these two transgenic approaches should inhibit β1 integrin signalling and disrupt myelination. The different outcomes from these three transgenic approaches can be explained by the existence of other laminin receptors essential for CNS myelination, which could reconcile the absence of a CNS phenotype in the β1 KO and also the different results shown in the dnβ1 and β1ΔC. One of the candidates for a laminin receptor would be dystroglycan, since several studies have suggested a role for dystroglycan in PNS myelination (Occhi *et al.,* 2005; Saito *et al.,* 2003). Dystroglycan has been reported to be expressed by oligodendrocytes and *in vitro* myelin formation is inhibited by dystroglycan siRNA (Colognato *et al.,* 2007; Wang *et al.,* 2007). Given this evidence, we propose that the discrepancy between the phenotypes of our dnβ1 and the β1ΔC is due to a requirement for dystroglycan in the latter in the later stages of myelin formation, with our dnβ1 showing a role for integrin during initiation of myelination.

The results reported in this chapter show that β1 integrin regulates the initial axoglial contact in myelination, with a delay in myelination of small diameter axons in dnβ1 mice. We also conclude that β1 integrin is not involved in myelin sheath formation or wrapping, since the internodal length and myelin thickness are normal in dnβ1 mice. Our findings thus suggest that β1 integrin is required to detect an axonal signal that induces myelination. The mechanism underlying this process could be explained by failure of oligodendrocyte differentiation, or a disturbance of the axoglial signalling that regulates initiation of myelination (Fig. 3.30).

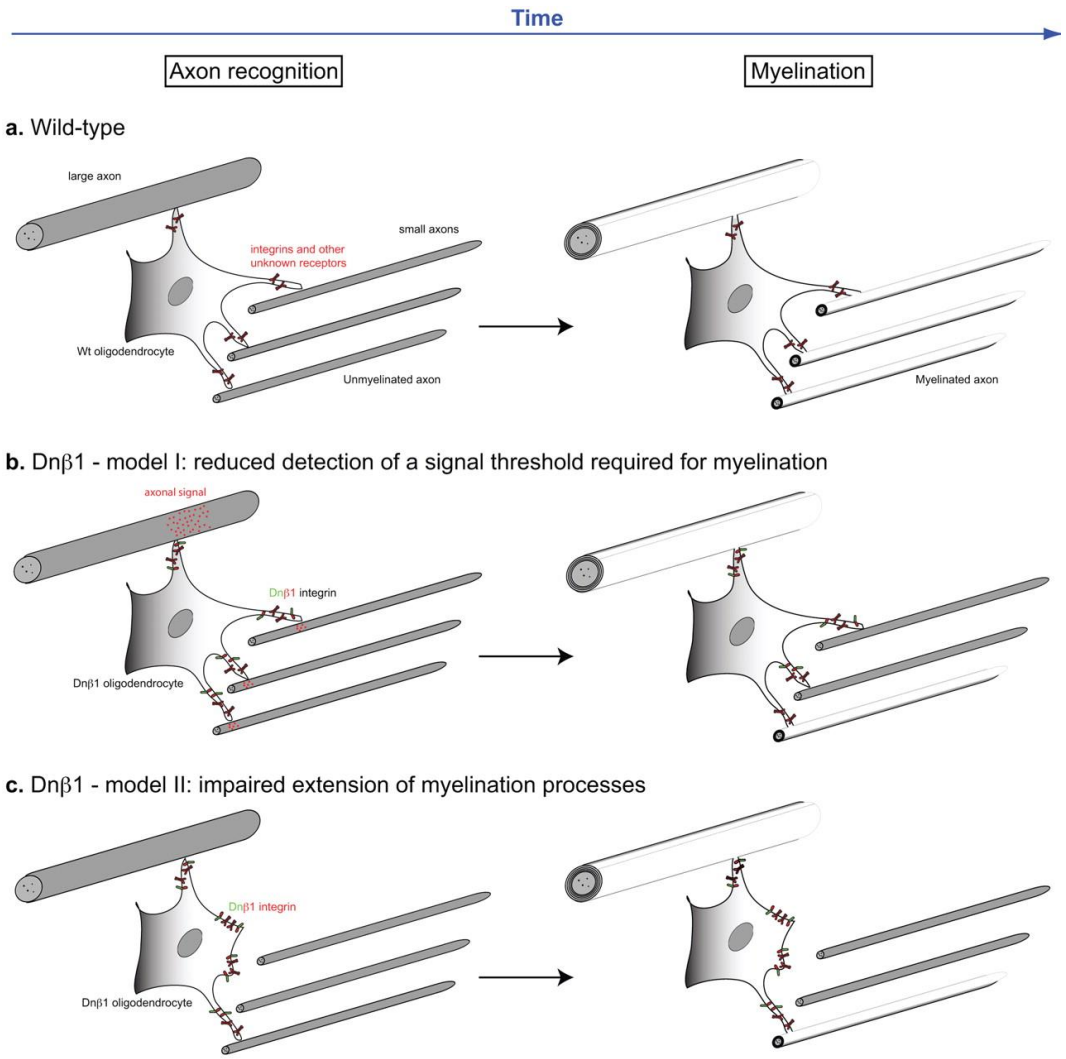


Figure 3.31 – Proposed mechanisms for delayed myelination of small-diameter axons in the dominant negative β1 integrin mice. Reproduced from Câmara *et al.*, 2009.

The first model assumes that reduction in β1 integrin signalling would preferentially affect small diameter axons, compared to large diameter axons, due to a failure of the oligodendrocyte to branch several processes for myelination. In agreement with this model, it has been shown that toad oligodendrocyte cell bodies associate closer to large fibres and have fewer processes than those related to small fibres (Hildebrand *et al.*, 1993; Stensaas and Stensaas, 1968). Further evidence is provided by Matthews and Duncan's observations that oligodendrocytes in rat white matter areas with both large and small diameter axons have fewer branches than areas composed of small axons only (Matthews and Duncan, 1971). It has also been suggested in the literature that ECM binding through β1 integrins and cytoskeletal reorganization are required

for the establishment of oligodendrocyte processes and myelin sheaths (Schoenwaelder and Burridge, 1999). To further support this view, a study of WAVE1 knockout mice showed that in the optic nerve and corpus callosum, these mice had a reduced number of myelinated axons, and the oligodendrocytes *in vitro* extended fewer processes than the wildtype (Kim *et al.,* 2006). Given the comparatively high number of processes required to reach small axons, a reduction in β1 integrin signalling would be expected to be particularly harmful to small axons. This evidence would suggest that disruption of β1 integrin signalling in our dnβ1 mice would lead to a decrease in the length of the internodes and a higher g-ratio (characteristic of hypomyelination). Our data goes against this hypothesis since *in vitro* the dnβ1 oligodendrocytes are able to myelinate normal internodal lengths, and the g-ratio at P17 is normal (Câmara *et al.,* 2009).

The second model proposed is that reduction of β1 integrin perturbs the signalling pathway that controls axoglial contact and initiation of myelination. When the oligodendrocyte contacts the axon it needs to detect a certain threshold level of β1 integrin expression, controlled by the axon diameter, in order to decide to myelinate it. If there is a reduction in the detector molecule (β1 integrin), the prediction would be that the small axons will be below the required level and a reduced percentage of myelinated axons will be observed. By contrast, the large axons are already above the required level and will not be affected. This hypothesis is consistent with what has been observed in the PNS, where neuregulins have been shown to play a crucial role in both decision of whether an axon gets myelinated and in the regulation of myelin thickness (Michailov *et al.,* 2004; Taveggia *et al.,* 2005). The neuregulin effect in CNS myelination is controversial, and seems rather more complicated. The addition of exogenous neuregulin to myelinating co-cultures of oligodendrocytes and dorsal root ganglion (DRG) neurons increases myelination (Wang *et al.,* 2007), whilst it is reduced when oligodendrocytes are cultured with neuregulin-deficient DRG neurons (Taveggia *et al.,* 2008). *In vivo* studies of neuregulins in the CNS are inconclusive, since mice haploinsufficient for neuregulin 1 type III show a hypomyelination phenotype (Roy *et al,* 2007; Taveggia *et al.,* 2008), but ablation of neuregulin 1, ErbB3 and ErbB4 (part of the ErbB receptor family that regulates

neuregulins) show no impairment in myelination (Brinkmann *et al.*, 2008). Together these results show that neuregulins may play a role in the CNS, but unlike in the PNS where neuregulins are individually responsible for myelination, in the CNS other molecules may be required.

Our lab has shown that integrins amplify neuregulin signalling. Integrin α6β1 is a laminin receptor that regulates oligodendrocyte survival signalling by amplification of neuregulin activity (Colognato *et al.*, 2002). We hypothesised that integrins and neuregulins act in concert and form a multi-component signalling complex responsible for initiation and regulation of myelination in the CNS. This complex could explain the results described in this chapter, where oligodendrocytes with reduced β1 integrin signalling would receive other cues in order to myelinate, and explain this transient phenotype.

The determination of the signals regulating the initiation of myelination is of the highest priority to promote the reset of the oligodendrocytes arrested at the premyelinating stage in the demyelinating disorder Multiple Sclerosis (Chang *et al.*, 2002; Kuhlmann *et al.*, 2008). In addition, the understanding of the mechanisms regulating myelination of small and large axons is crucial to determine why in Krabbe's disease, late myelinated tracts are preferentially affected in contrast to early myelinated tracts. The biological reason for this could lie in the differential susceptibility of large and small axons to disruption of different signalling molecules.

Chapter 4

Role of β1 integrin and neuregulin 1 in central nervous system myelination

Chapter 4

4.1 Introduction

Myelination is a tightly controlled process where a glial cell wraps several layers of myelin around an axon in order to insulate it, improve conduction performance, and provide long term axonal integrity (Griffiths *et al.,* 1998; Huxley and Stampfli, 1949). Central nervous system (CNS) and peripheral nervous system (PNS) myelination differ in terms of the cells responsible for myelination (oligodendrocytes and Schwann cells, respectively) and some of the core proteins that constitute the myelin layer (PLP in the CNS vs P0 in the PNS). Oligodendrocytes can extend several processes and myelinate different axons, while Schwann cells can only myelinate one internode. For myelination to occur, a glial cell needs to establish contact with an axon and identify the number of wraps that will provide complete insulation of the axon. The thickness of the myelin sheath is regulated by the axon diameter (Friede, 1972) with an axonal threshold of 0.2 μm in the CNS and 1 μm in the PNS (Waxman and Bennet, 1972; Voyvodic, 1989) necessary for myelination to occur. The existence of a threshold for myelination together with the observation that *in vitro* oligodendrocytes are able to extend processes in the absence of neurons, but can only form compact myelin layers in the presence of axons, shows that axonal signalling and/or secreted molecules are necessary for initiation of myelination (Lubetski *et al.*, 1993). Furthermore, transplantation of oligodendrocytes from the optic nerve (an area characterized by small calibre axons) into the spinal cord (with typically large diameter axons) leads to the correct amount of myelin sheaths being formed, showing that myelin thickness is not an intrinsic property of the oligodendrocyte, but regulated by the axon (Fanarraga *et al.,* 1998).

In the PNS, the levels of neuregulin 1 type III on the surface of the axon have been identified as a necessary and sufficient regulator for myelination. In the CNS, such a signal has yet to be identified. Work described in chapter 3 identified β1 integrin as a

regulator for axoglial contact, and we hypothesized that β1 integrin acts in concert with other signalling molecules, such as neuregulin 1 type III, to control myelin thickness in the CNS.

Central hypothesis

The central hypothesis of this project is that neuregulins act in concert with β1 integrin to regulate the axoglial signalling necessary for the initiation of myelination

Statement of aims

1. Generate transgenic mice by crossing male dominant negative β1 integrin mice with neuregulin 1 type III heterozygous females
2. To characterize myelination in the dominant negative β1 integrin // neuregulin 1 type III $^{+/-}$ mice by analysing g-ratio and percentage of myelination by electron microscopy.at different time points.and different structures of the brain.

4.1.2 Neuregulins: structure and function

The neuregulins are a family of growth and differentiation factors that mediate cell-cell interactions in the nervous system, heart and breast (Falls, 2003). Investigations by two different groups identified neuregulin as a ligand for the ErbB2 receptor (Holmes *et al.,* 1992; Peles *et al.,* 1992; Wen *et al.,* 1992); at the same time, a glial growth factor (GGF) that stimulates Schwann cell proliferation was also identified (Goodearl *et al.,* 1993; Marchionni *et al.,* 1993), as was a growth factor (ARIA) that stimulates acetylcholine receptor synthesis (Falls *et al.,* 1993). It was later found that all of these proteins are encoded by the *NRG1* gene (Falls, 2003). The neuregulin family comprises 4 distinct genes that encode neuregulin 1 (Nrg1), neuregulin 2 (Nrg2) (Busfield *et al.,* 1997; Carraway *et al.,* 1997; Chang *et al.,* 1997; Higashiyama *et al.,* 1997), neuregulin 3 (Nrg3) (Zhang *et al.,* 1997) and neuregulin 4 (Nrg4) (Harari *et al.,* 1999). They share an EGF-like domain essential for receptor

binding and activation. The biological functions of Nrg2, Nrg3 and Nrg4 have not been fully identified, and for the purposes of this thesis the focus will be on Nrg1.

Nrg1 is encoded by a single gene comprising approximately 1.4 megabases. Through alternative splicing and multiple promoters, this gene encodes more than 30 different isoforms in humans. These were then subdivided into type I-VI, according to their membrane topologies (Mei and Xiong, 2008). All isoforms contain an epidermal growth factor (EGF)-like domain located in the extracellular region that is necessary and sufficient for activation of their receptors. As mentioned previously, throughout the years several proteins were identified such as neu differentiation factor (NDF), heregulin and acetylcholine receptor inducing activity (ARIA), that are now known to be part of the Nrg1 family and belong to *type I* Nrg1. The type I comprises an immunoglobulin (Ig)-like domain and a glycosylated domain. Their EGF-like domain can either be an α- or β-variant, which vary in their affinity to the receptor. *Nrg1 type II*, also termed glial growth factor (GGF), presents an Ig-like domain and a β-variant EGF-like domain. Its N-terminal comprises a classic signal peptide and a kringle-like domain. The kringle domain is responsible for mediation of protein-protein interactions in other proteins, but thus far there are no known partners for Nrg1 type II. *Nrg1 type III* is the most abundant Nrg1 in the nervous system and its expression is mostly restricted to neurons. It is also called sensory and motor neuron derived factor (SMDF) and it has a cystein-rich domain (CRD) with a hydrophobic domain on the NH_2-terminus and its EGF-like domain is β-variant. Both N- and C-terminals are located inside the cell since this isoform has a hydrophobic anchor sequence, giving this isoform a hairpin-shape membrane conformation (Fig. 4.1).

Ngr1 isoforms I-III have different expression patterns, with Nrg1 type I being preferentially expressed during early embryogenesis, whilst Nrg1 type II is expressed during nervous system late development and after birth, and Nrg1 type III is predominantly expressed by sensory and motor neurons (Meyer and Birchmeier, 1994; Meyer *et al.*, 1997).

In recent years Nrg1 *types IV-VI* have been described. These isoforms all share the EGF-like and Ig-like domain, with type VI having its N-terminal specific region directly associated with the EGF-like domain. Not much is known about the processing of these proteins but it is thought that this process is similar to the processing of types I and II.

Nrg 1 type I-III are considered the "classic" neuregulins (present in all vertebrates) while type IV-IV are considered "new" (restricted to primates) (Talmage, 2009). Not much is known about the function of these "new" neuregulins, so the main focus of this chapter will be on the "classic" neuregulins, more specifically on Nrg1 type III. Nrg 1 type IV has recently been implicated in schizophrenia and its role in mental disorders will be further discussed in Chapter 5.

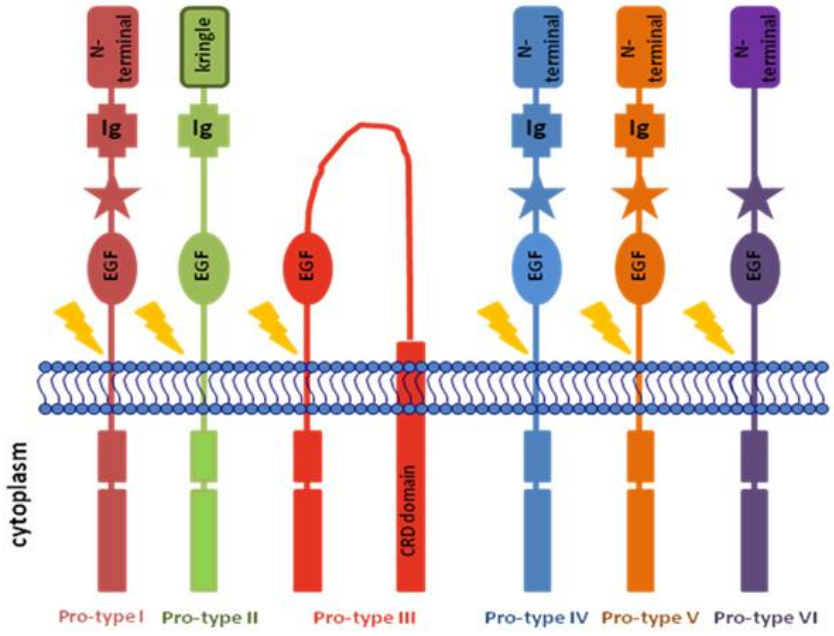


Figure 4.1 – Neuregulin 1 and respective isoforms. The different isoforms of neuregulin 1 (Type I-IV) all share an EGF-like domain in the extracellular region and are classified according to their type specific sequences. Neuregulin 1 isoforms are synthesised as transmembrane proteins, and after proteolitic cleavage are shed and released as soluble factors (type I-II and IV-VI) except in the case of type III which has both the N- and C-terminal inside the cell. Stars: glycosalytion. Lightning: proteolitic cleavage.

Neuregulins are responsible for multiple functions and have a role in different organs of the organism. Different *in vivo* and *in vitro* approaches have suggested a critical role for neuregulins in cardiac development, neural development, mammary gland development and muscle metabolism. Neuregulin 1 has been associated with survival, proliferation, differentiation and myelination in Schwann cells. In oligodendrocytes, neuregulin 1 affects proliferation, survival, differentiation and myelination. The initial formation of the nerve muscle interaction in the neuromuscular synapse is also controlled by Nrg1, as is the muscle spindle development, and also regulates neuronal neurotransmitter receptors, amongst other functions (reviewed by Falls, 2003).

4.1.3 Neuregulin 1 signalling and the nervous system

ErbB receptor family

The ErbB receptor tyrosine kinase is composed of four structurally related tyrosine kinases: ErbB1 (or HER1, or EGFR), ErbB2 (or neu in rodents, HER2 in humans), ErbB3 (or HER3) and ErbB4 (or HER4). All four tyrosine kinases share an extracellular ligand-binding region (or ectodomain with approximately 620 amino acids), a single transmembrane-spanning region and a cytoplasmic protein tyrosine kinase-containing domain (Holbro and Hynes, 2004). The ErbB family is expressed in several tissues through development and is involved in many cellular processes such as cell growth, division, migration, adhesion and apoptosis (Gerbin, 2010). ErbB receptor activation is spatially and temporally regulated by its ligands. ErbB ligands (EGF-related peptide growth factors) are synthesised as transmembrane precursors, their ectodomains are shed by proteolysis, responsible for soluble factor formation (Massagué and Pandiella, 1993). In mammals, ligand binding specificity is achieved by an EGF-like domain present in the ligands. There are 3 groups of ErbB ligands: 1) EGF, transforming growth factor-α (TGFα) and amphiregulin bind to ErbB1; 2) betacellulin, heparin-binding EGF (HB-EGF) and epiregulin which bind both ErbB1 and ErbB4; and 3) neuregulins, with two subgroups depending of their

ability to bind ErbB3 and ErbB4 (Nrg1 and Nrg2) or ErbB4 only (Nrg3 and Nrg4). So far no ligand for ErbB2 has been identified (Fig. 4.2) (Holbro and Hynes, 2004).

Ligand binding to ErbB receptors induces homo- or heterodimerization and subsequent activation of the intrinsic kinase domains leading to specific tyrosine residues to be phosphorylated (which act as docking sites for ligands) in the cytoplasmic domain, which in turn activates the intracellular signalling pathways.

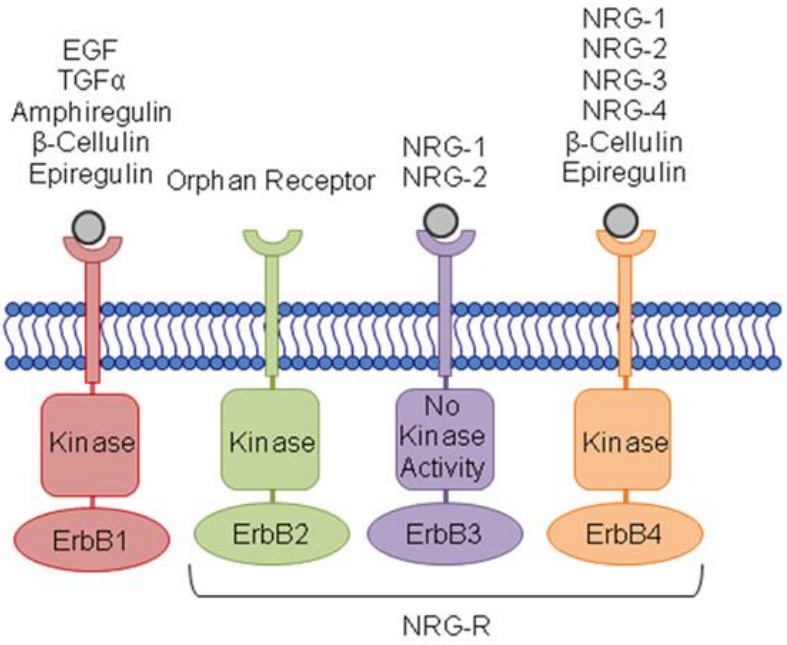


Figure 4.2 – ErbB receptors and its ligands. Structure of the four tyrosine kinases (ErbB1, ErbB2, ErbB3 and ErbB4) and their respective ligands. ErbB receptors share an extracellular ligand-binding region (or ectodomain with approximately 620 amino acids), a single transmembrane-spanning region and a cytoplasmic protein tyrosine kinase-containing domain. Note that ErbB3 lacks tyrosine kinase and needs to heterodimerize with other ErbBs in order to be activated.

The extracellular region of the ErbB receptor is composed of four domains (I-IV). ErbB1 inactive structure presents an intramolecular interaction between domains II and IV which mediates receptor-receptor interaction. Since the domain II and IV are located in the same dimerization arm, this confers the ErbB1 a closed conformation. In order to achieve an open conformation the ErbB1 domain needs to undergo rearrangement (Fergurson *et al.*, 2003; Cho and Leahy, 2002). Interestingly, ErbB2 is always in a conformation that resembles the ligand-activated ErbB1, with no

interaction between the II-IV domains and an exposed dimerization loop. This may explain why ErbB2 is an orphan receptor, since the proximity of the domains I and III prevents EGF-related peptides ligation. ErbB3 lacks tyrosine kinase activity and in its nonactivated state has an analogous conformation to ErbB1. In order to be phosphorylated, ErbB3 needs to heterodimerize with other ErbB receptors. ErbB4 comprises a functional ligand-binding domain and a tyrosine kinase domain, allowing ErbB4 to bind neuregulin as a homodimer (Bao *et al.*, 2003)

In the context of the work described in this thesis, the main focus will be on ErbB/Nrg1 signalling.

ErbB/Nrg1 signalling

The EGF-like domain present in the extracellular domain of neuregulins interacts and activates the ErbB receptor and stimulates the different signalling pathways. This is accomplished after proteolytic cleavage by 3 different type I membrane proteases: tumour necrosis factor-α converting enzyme (TACE/ADAM17), β-site of amyloid precursor proteins cleaving enzyme (BACE or also termed memapsin2) or meltrin-β (ADAM19). After proteolytic catalysis, the EGF-like domain is released and binds specific receptors to initiate autocrine or paracrine/juxtacrine signalling (Goodearl *et al.*, 1995; Loeb *et al.*, 1998). Neuregulin 1-type III comprises a CRD-domain which remains attached to the membrane after proteolytic cleavage and presents an extra binding point, therefore conferring these isoform properties for juxtacrine signalling. By contrast, the other Nrg1 isoforms are shed and released as soluble growth factors after cleavage and act as paracrine signalling molecules (Gumà *et al.*, 2010).

Activation of ErbB receptors occurs via dimerization mediated by their ligands (Schlessinger, 2000). Nrg1 is unable to bind ErbB1. Although Nrg1 only binds ErbB3 and ErbB4, Nrg1 can, in theory, interact with all four ErbB receptors by inducing formation of heterodimers (Murphy *et al.*, 2002). Since ErbB2 lacks a binding domain but has an active catalytic protein tyrosine kinase, its activation is achieved by heterodimerization with the ErbB3 and ErbB4 receptors (Graus-Porta *et*

al., 1997). ErbB3 is deprived of tyrosine kinase activity, therefore functioning as a signalling partner only when dimerized. ErbB3 homodimers can bind to Nrg1 but are catalytically inactive (Guy *et al*, 1994). In contrast, ErbB4 comprises a ligand-binding and a tyrosine kinase domain, which allows the formation of homodimers capable of Nrg1 binding (Gumà *et al.*, 2010). Nrg1 interacts with the homodimer Erb4/ErbB4 and the heterodimers ErbB2/ErbB3, ErbB2/ErbB4 and ErbB3/ErbB4 (Mei and Xiong, 2008). The ability to form different homo- and heterodimers explains the importance of ErbB receptors in different organs since they can interact with a multitude of ligands, and therefore regulate several signalling pathways.

Nrg1/ErbB signalling activates extra- and intracellular pathways. After Nrg1-ErbB binding, the ErbB receptor undergoes conformational changes and dimerizes with another ErbB receptor. Dimerization activates the tyrosine kinase and induces tyrosine auto- and trans-phosphorylation of the cytoplasmic domain of the receptors, which causes the exposure of binding sites for molecules that regulate downstream signalling (such as grb2, grb7, PLCγ , shc or p85) (Burden and Yarden, 1997; Yarden and Sliwkowski, 2001). ErbB/Nrg1 signalling regulates the MAPK and the PI3K/Akt signalling pathways (Yarden and Sliwkowski, 2001, Zorzano *et al.*, 2003) that induce cellular responses such as migration, proliferation, differentiation, survival and apoptosis (Lemke, 1996; Burden and Yarden 1997; Adlkofer and Lai, 2000; Garratt *et al.*, 2000; Buonanno and Fischbach 2001; Yarden and Sliwkowski 2001; Citri *et al.*, 2003; Falls 2003).

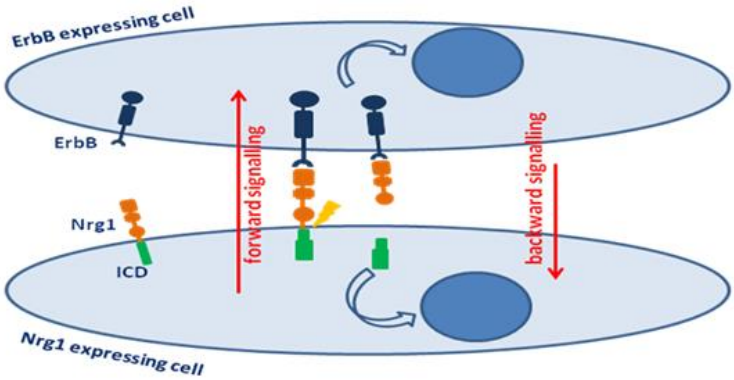


Figure 4.3 – Bidirectional signalling by neuregulin 1. Interactions between ErbB receptors and neuregulins results in both forward and backward signalling. In forward signalling, ErbB cleavage by γ-secretase results in the release of the ErbB-intracellular domain that can translocate to the nucleus to regulate gene expression in ErbB expressing cells. In backward signalling, extracellular cleavage releases the C-terminal of neuregulins which can be further cleaved by γ-secretase and generates the intracellular domain that will translocate to the nucleus and regulate gene expression. Blue circles represent the nucleus.

Signalling by Nrg1/ErbB can be divided into forward and backward signalling (Fig. 4.3), with forward signalling being either canonical or non-canonical. In canonical forward signalling, ErbB is phosphorylated after ligand binding, and as a consequence, docking sites for phosphatase-binding adaptor molecules are exposed, which results in ErbB internalization (Gu *et al.*, 2005; Liu *et al.*, 2007; Yang *et al.*, 2005). This process activates several signalling pathways such as Raf-MEK-Erk and PI3K-Akt-S6K, with downstream kinases including c-Abl, JNK, Fyn, CDK5 and Pyk2 (Bjarnadottir *et al.*, 2007; Fu *et al.*, 2001; Si *et al.*, 1999; Yarden and Sliwkowski, 2001). The wide spectrum of pathways affected by Nrg1/ErbB signalling explains the diversity of biological functions regulated by Nrg1.

Non-canonical forward signalling is dependent on ErbB and its subsequent cleavage. Initial cleavage of the ErbB4 juxtamembrane by TACE exposes a binding site (ecto-ErbB4) for Nrg1 after release of a soluble extracellular peptide which in turn leads to cleavage of the ErbB4 transmembrane anchored domain by presenilin-dependent γ-secretase, followed by release of the intracellular domain of ErbB4 (Lee *et al.*, 2002; Ni *et al.*, 2001). Transcription is regulated by translocation of the intracellular domain to the nucleus (Sardi *et al.*, 2006).

Nrg1 isoforms are synthesized as pro-Nrg1. They are transmembrane proteins that, after ligation with a receptor, are either secreted as soluble factors (type I-II and type IV-VI) or shed and remain attached to the membrane (type III). The membrane-associated Nrg1 isoforms can be shed into extra- or intracellular space since they have internal proteolytic cleavage sites (Bao *et al.*, 2003).

Backward signalling is based on the property of Nrg1 acting as receptors and regulating signalling. The first mechanism proposed for backward signalling is based on pro-Nrg1 type I-II/IV-VI, where after activation of pro-Nrg1 and extracellular cleavage, the intracellular region can be further cleaved by γ-secretase. The cleavage of the Nrg1 intracellular domain allows it to be translocated to the nucleus and regulate gene transcription. Another mechanism proposed for backward signalling consists on the properties of the ErbB4 or the ecto-ErbB4 to act as a ligand for Nrg1, and therefore activate downstream pathways. So far, the molecules that regulate the nuclear signalling pathways in this model have not been identified. Therefore ErbB/Nrg1 signalling is a complex system with Nrg1 functioning as either a ligand for ErbBs in forward signalling or a receptor for ecto-ErbB4 in backward signalling (Bao *et al.*, 2003; Mei and Xiong, 2008).

Neuregulin 1 and the nervous system

Neuregulin 1 and its receptor ErbB regulate multiple cellular functions and its importance is shown by transgenic Nrg1- and ErbB-null mice that are embryonically lethal (E10.5-13.5) (Erickson *et al.*, 1997; Gassmann *et al.*, 1995; Kramer *et al.*, 1996; Lee *et al.*, 1995; Meyer and Birchmeier, 1995; Wolpowitz *et al.*, 2000).

In the nervous system, Nrg1 has a role in functions such as: radial neuron migration, axon guidance, myelination and ensheathment in the PNS, oligodendrocyte development, neuromuscular junction (NMJ) formation and synapse formation (Fig. 4.3) (Mei and Xiong, 2008). A brief description of how Nrg1 controls some of these processes will follow with a more detailed explanation of the specific effects of Nrg1 type III in myelination of the PNS and CNS.

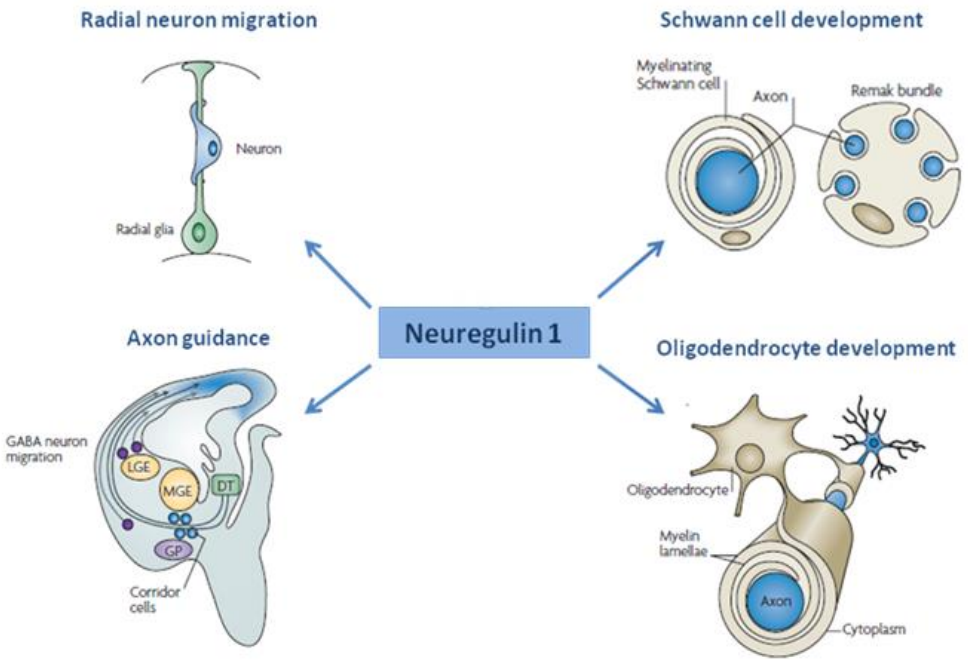


Figure 4.4 – Distinct roles of neuregulin 1 in the central nervous system. The different types of neuregulin 1 have been described to play a myriad of roles in both the peripheral and central nervous system, such as modulating Schwann cell development, nerve repair and remyelination. Neuregulin 1 also plays a role in fundamental processes of the brain such as neuronal migration and axon guidance. During **o**ligodendrocyte development, neuregulin 1 influences the migration and differentiation of oligodendrocyte precursor cells. Since neuregulin 1 is involved in so many aspects of nervous system development there is an increased interest to delve in its role in myelination. Adapted from Mei and Xiong, 2008.

Radial neuron migration and axon guidance

Neural crest cells are a pluripotent embryonic cell population that arise from the dorsal neural tube (Le Douarin *et al.*, 2004) and undergo an epithelial-mesenchymal transition, followed by detachment of the neural tube epithelium. These cells then migrate substantial lengths to reach specific target sites and differentiate into a distinct number of cells that form the peripheral nervous system (such as neuronal and glial cells), skin (melanocytes), facial bone and cartilage, and heart (Anderson *et al.*, 1997; Groves and Bronner-Fraser, 1999; Crane and Trainor, 2006). Nrg1 is expressed in the dorsal neural tube and along the migratory paths and acts as a chemoattractive factor for migratory neurons. Nrg1/ErbB signalling regulates

tangential migration of GABAergic neurons and radial migration of glutamatergic neurons. Radial glial cells express ErbB2 and ErbB3 while neurons express ErbB3 and ErbB4. *In vitro*, neural migration along radial fibres is inhibited when glial cells express a dominant negative ErbB4 (Rio *et al.*, 1997). In tangential migration of interneurons to the cortex, a subset of interneurons expresses ErbB4 and migrates along an Nrg1 type III-expressing corridor (Flames *et al.*, 2004; Yau *et al.*, 2003). Nrg1 controls short and long range migration of cortical GABAergic interneurons, with loss of ErbB4 resulting in a decreased number of interneurons in the cortex (Flames *et al.*, 2004). Neurite outgrowth in the hippocampus, retina, thalamus and cerebellum is stimulated by recombinant Nrg1 (Bermingham-McDonogh *et al.*, 1996; Gerecke *et al.*, 2004; Lopez-Bendito *et al.*, 2006; Rieff *et al.*, 1999). The exact mechanism of action of Nrg1 in axonal guidance is not fully understood since Nrg1 knockout mice die at postnatal day 10.5 (P10.5).

Schwann cell and oligodendrocyte development

Schwann cells derive from the neural crest. Neural crest cells differentiate into myelinating or nonmyelinating Schwann cells after migrating through sensory and motor Nrg1 expressing neurons (Meyer *et al.*, 1997). Nrg1 was found to be a key regulator of Schwann cell development, regulating the glial fate of the neural crest cells (Lai and Feng, 2004), proliferation (Dong *et al.*, 1995; Grispan *et al.*, 1996; Marchionni *et al.*, 1993; Schmid *et al.*, 2003; Winseck *et al.*, 2002; Wolpowitz *et al.*, 2000) and migration of the neural crest cells (Mahanthappa *et al.*, 1996; Wolpowitz *et al.*, 2000). Mutant mice for Nrg1, ErbB2 and ErbB3 all show developmental abnormalities in neural crest cells (Riethmacher *et al.*, 1997). Once neural crest cells have differentiated into Schwann cells, their proliferation and migration, differentiation and survival is also regulated by Nrg1 (Scherer *et al.*, 1995; Trachtenberg and Thompson, 1996; Winseck *et al.*, 2002). *In vitro*, Schwann cells supplemented with Nrg1 actively proliferate, contrasting with what is seen when Schwann cells are cultured with serum present (Brockes *et al.*, 1980; Lemke and Brockes, 1984). ErbB3 mutant mice show no heart abnormalities and after analysis of the peripheral nervous system, a significant decrease in the number of Schwann cells was observed (Riethmacher *et al.*, 1997). Nrg1 and ErbB2 null mice die before

birth due to heart defects, a transgenic approach where the heart abnormalities are rescued by heart-specific expression of ErbB2 allowed proper investigation into the Nrg1/ErbB role in the PNS. These mice show a similar phenotype to the ErbB3 mutant, lacking Schwann cells and having defasciculated nerve bundles (Woldeyesus *et al*., 1999).

Interestingly, the ErbB mechanism that regulates Schwann cell development is conserved in zebrafish. Due to the intrinsic characteristics of zebrafish, it is possible to observe their embryonic development and perform time-lapse analysis *in vivo*. Zebrafish mutants for ErbB2 and ErbB3 present a similar phenotype seen in mutant mice (Honjo *et al.,* 2008; Lyons *et al.,* 2005). Impaired migration of Schwann cell precursors along the developing axon and reduced proliferation rates were observed in the ErbB3 mutant embryo. After chemical inhibition of ErbB, it was also reported that Schwann cell proliferation is disrupted and these cells show an aberrant migratory pattern (Lyons *et al.,* 2005).

Nrg1 and ErbBs are widely expressed in neurons and oligodendrocytes of the developing and adult CNS (Meyer *et al.,* 1997; Buonanno and Fishbach, 2001; Falls, 2003; Anton *et al.,* 2004). Due to the early lethality of Nrg1 and ErbB mutant mice, it is difficult to assess their function during CNS development since the developing process of the CNS is not constrained to the embryonic period and lasts until after birth. Therefore most data that suggest a role for Nrg1 in CNS development was generated mainly from *in vitro* and *ex vivo* studies. *In vitro*, Nrg1 was shown to have a role in oligodendrocyte specification, proliferation, differentiation and survival (Calaora *et al.,* 2001; Canoll *et al.,* 1996; Canoll *et al.,* 1999; Flores *et al.,* 2000; Vartanian *et al.,* 1997). Vartanian and co-workers showed that Nrg1 knockout mice fail to generate oligodendrocytes after examining cultures of spinal cord explants from these mice. This phenotype can be rescued by exogenous Nrg1 (Vartanian *et al.,* 1999). In the chicken model, the spinal cord is deprived of oligodendrocyte precursors during normal development. Explants from dorsal spinal cord supplemented with sonic hedgehog and Nrg1 can give rise to oligodendrocyte progenitors (Sussman *et al.,* 2000). *In vivo*, ErbB2 has also been shown to affect

oligodendrocyte development. Dominant negative ErbB2 mice show an increase in oligodendrocyte precursor cells and a reduction of myelinating oligodendrocytes (Kim *et al.,* 2003).

Neuregulin 1 and myelination – PNS vs. CNS

Glial cells of the nervous system are responsible for ensheathing axons with a specialized plasma membrane – myelin – in order to electrically insulate the axon and allow a faster propagation of the nervous impulse. Schwann cells in the PNS and oligodendrocytes in the CNS myelinate axons using different processes. While Schwann cells establish a 1:1 ratio with axons, oligodendrocytes are capable of myelinating multiple axons of different sizes showing that axons play an important role in the regulation of myelin thickness (Baumann *et al.*, 2001). Friede showed that myelin thickness is proportional to axon diameter. This relation is termed the g-ratio and is the ratio between the radius of inner and outer circumferences of the myelin sheath (Fig.4.5) (Friede, 1972). Nrg1 is expressed on the axon surface and the specific Nrg1 type III isoform has been shown to be a key regulator of peripheral myelination.

More than a decade ago, it was initially proposed that Nrg1 and its receptors played a role in peripheral myelination. Mice with null mutations for Nrg1, ErbB2 or ErbB3 show aberrant Schwann cell development. Since ErbB2 knockout mice are embryonic lethal, Garratt and co-workers applied a conditional inactivation of ErbB2 strategy specific to Schwann cells, with mice exhibiting a hypomyelination phenotype (Garratt *et al.,* 2000). Further transgenic approaches were taken to assess the regulation of myelination and myelin thickness. Comparison of wildtype mice and $Nrg1^{+/-}$, $Nrg1^{+/-}$//$ErbB2^{+/-}$ and $Nrg1^{+/-}$//$ErbB2^{+/-}$//$ErbB3^{+/-}$ showed that all these mice share the same phenotype with decreased myelin thickness (Michailov *et al.,* 2004). Accordingly, axons that over-express Nrg1 type III have thicker myelin, demonstrating that myelin wrapping is proportional to Nrg1 expression levels (Fig. 4.5) (Michailov *et al.,* 2004). In the PNS, Nrg1 contributes to the initiation of myelination. Dorsal root ganglion (DRGs) isolated from mice with reduced Nrg1

type III expression failed to be myelinated in culture, even when the number of Schwann cells available was increased five-fold. This phenotype can be rescued by lentiviral expression of Nrg1 type III. Superior cervical ganglia are characterized by small caliber axons and express low levels of Nrg1 type III, and therefore are not myelinated *in vivo*. Lentiviral expression of Nrg1 type III on the surface of these axons is sufficient to promote myelination (Taveggia *et al.*, 2005).

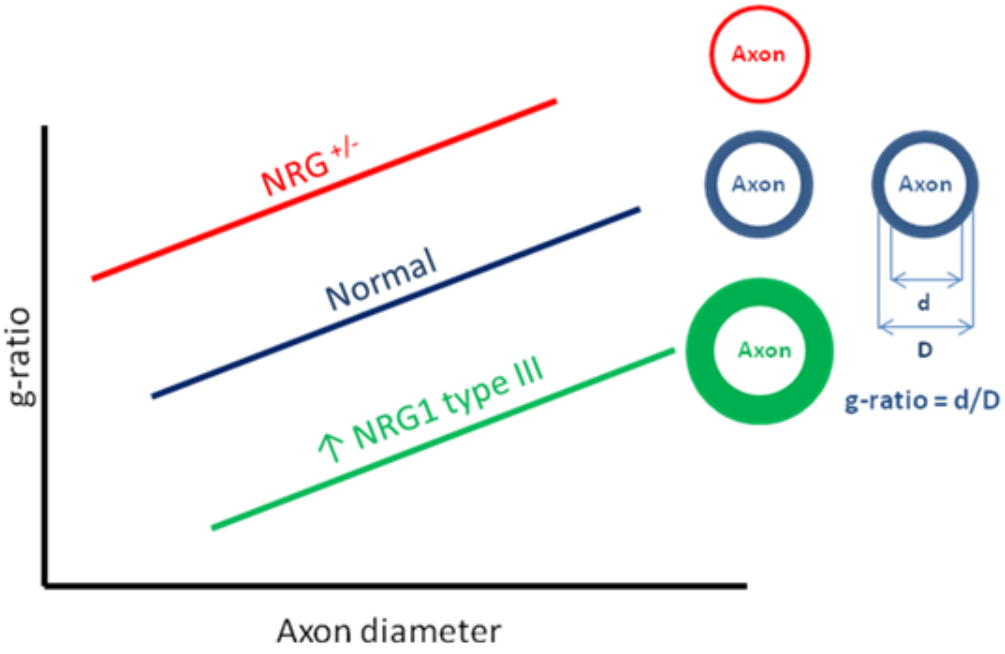


Figure 4.5 – Neuregulin 1 controls myelin thickness in the peripheral nervous system. Variation in expression of axonal neuregulin 1 regulates the myelin thickness in the PNS, with heterozygous mice for Nrg1 showing hypomyelination and overexpression of Nrg1 leading to hypomyelination.

Contrary to the PNS, the role of Nrg1 in the CNS is not well established. One study that can support the view that Nrg1 also plays a role in central myelination used the beta-site amyloid precursor protein-cleaving enzyme 1 (BACE 1) knockout mice. Bace1 modulates myelination both in the PNS and the CNS, and the Bace1 KO mice showed hypomyelination and decreased myelin thickness (Hu *et al.*, 2006). *In vitro*, the addition of exogenous Nrg1 to myelinating co-cultures of oligodendrocytes with DRG neurons showed an increase in myelination (Wang *et al.*, 2007), and in co-cultures of Nrg1 type III-deficient DRG neurons myelination was reduced (Taveggia *et al.*, 2008). *In vivo*, transgenic mice expressing a dominant-negative ErbB receptor

in oligodendrocytes and mice haplosuffficient for Nrg1 type III showed hypomyelination (Taveggia *et al.*, 2008; Roy *et al.*, 2007).

It was therefore likely that neuregulin played a similar role in the CNS to that already described in the PNS. However, overexpression of Nrg1 does not promote myelination of small diameter and normally unmyelinated axons in co-cultures of superior cervical ganglion neurons and oligodendrocytes (Taveggia *et al.*, 2008). It was also reported that conditional mutants with ablation of Nrg1, ErbB3 or ErbB4 had no cortical myelination abnormalities (Brickmann *et al.*, 2008), suggesting that Nrg1 signaling it is not required for CNS myelination.

Integrins amplify neuregulin signalling

Integrins, the major family of extracellular matrix (ECM) receptors are involved in the regulation of many fundamental cellular functions such as proliferation, survival, and migration (reviewed by Laursen and ffrench-Constant, 2007). Integrins are heterodimers of an α and β chain that bind the ECM ligands and signal through pathways including PI3K and MAPK (Hynes, 2002). Interaction with a wide range of receptors including growth factor receptors (such as the ErbB receptor family that regulates neuregulin) is well described (reviewed by ffrench-Constant, 2004). One of these integrins, α6β1, binds laminins expressed around axons at the time of myelination and is known to regulate oligodendrocyte survival signalling by amplification of neuregulin activity (Colognato *et al.*, 2002).

In the CNS, growth factors can regulate cell proliferation, survival and differentiation in a context-dependent manner. In the course of oligodendrocyte development, differentiation and survival are regulated by axonal contact and soluble factors (PDGF and Nrg1) (Barres and Raff, 1999; Buonanno and Fischbach, 2001). These factors have contradictory effects throughout oligodendrocyte development, acting as mitogens and differentiation inhibitors in early stages, and in later stages cease their mitogenic and inhibitory function and switch to promote oligodendrocyte survival (Colognato *et al.*, 2002). This switch can be explained through integrin signalling

and its properties regulating growth factor responses. Integrin α6β1 is one of the heterodimers expressed by oligodendrocytes and mediates neuronal survival signalling in oligodendrocytes. α6β1 integrin binds laminin-2 which is expressed by axons during myelination and this interaction is known to regulate survival of oligodendrocytes that contact axons by reducing the threshold requirement for PDGF and Nrg signalling. Integrin α6-null mice die at birth but myelination of axon tracts of the brainstem and cervical spinal cord is initiated before birth which can be used for the analysis of α6 integrin function. In cultures from integrin α6-null mice, a decrease in MBP positive cells and an increase in apoptosis were observed when compared to wildtype. Axons derived from the integrin α6-null mice were cultured in laminin-2 and supplemented with neuregulin switch from a PI3K to a MAPK pathway (showed by an increased MAPK phosphorylation). PI3K promotes proliferation and survival but inhibits differentiation. MAPK promotes differentiation and survival. This switch to MAPK-dependent signalling enhances survival and presents a mechanism for axon-mediated survival of oligodendrocytes regulated by integrins through axonal neuregulin.

4.2 Results

4.2.1 Analysis of dominant negative β1 integrin // neuregulin 1 type III $^{+/-}$ mice

As described in Chapter 3, dominant negative β1 integrin (dnβ1) mice showed that integrins mediate the axoglial interactions in myelination (Câmara *et al.*, 2009). These mice reduce β1 integrin signalling independently of ligand binding. To test our hypothesis, that β1 integrin acts in concert with neuregulin 1 to regulate the initial axoglial contact, we crossed the dnβ1 mice with neuregulin 1 type III heterozygous (neuregulin 1 type III $^{+/-}$) mice. These mice were a kind gift from Dr. Carla Taveggia and have been described elsewhere (Wolpowitz *et al.*, 2000). The offspring was either dnβ1 // neuregulin 1 type III$^{+/+}$ (used as a control) or dnβ1 // neuregulin 1 type III$^{+/-}$. Neuregulin 1 type III haploinsufficient mice showed no defect in myelination

in the optic nerve and spinal cord but showed hypomyelination of the corpus callosum (Taveggia *et al.*, 2007).

To analyse myelin integrity in the dnβ1 // neuregulin $1^{+/-}$ mice, we initially performed ultrastructural electron microscopy (EM) analysis and measured the ratio between the radius of the inner and outer circumferences of the myelin sheath (g-ratio) on optic nerves of mice at postnatal day 17 (P17), a time at which myelination is not yet complete (Figure 4.6). Approximately 100 axons from each sample were analysed, in 4 animals of dnβ1//neuregulin $1^{+/+}$ (n = 400 axons), with an average g-ratio of 0.82 ± 0.014 in dnβ1//neuregulin $1^{+/-}$ mice (n = 596 axons, 6 animals) and an average g-ratio of 0.82 ± 0.00 in dnβ1//nneuregulin $1^{+/+}$, with no difference (P=0.76) observed between genotypes (Fig. 4.6).

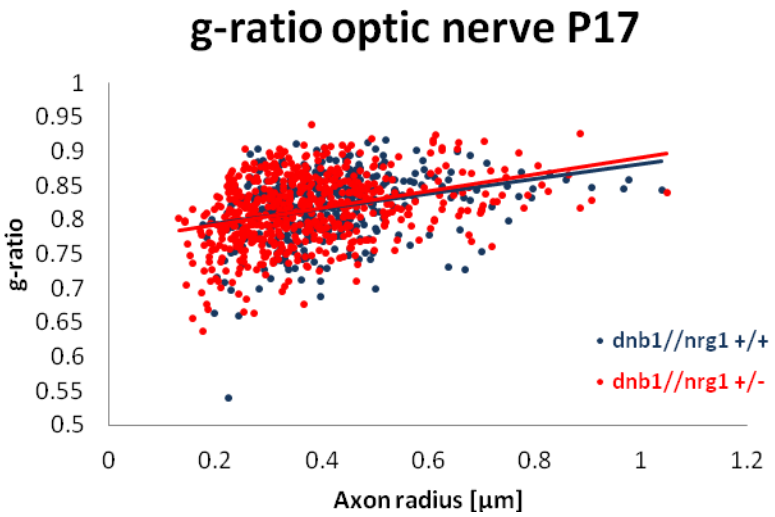
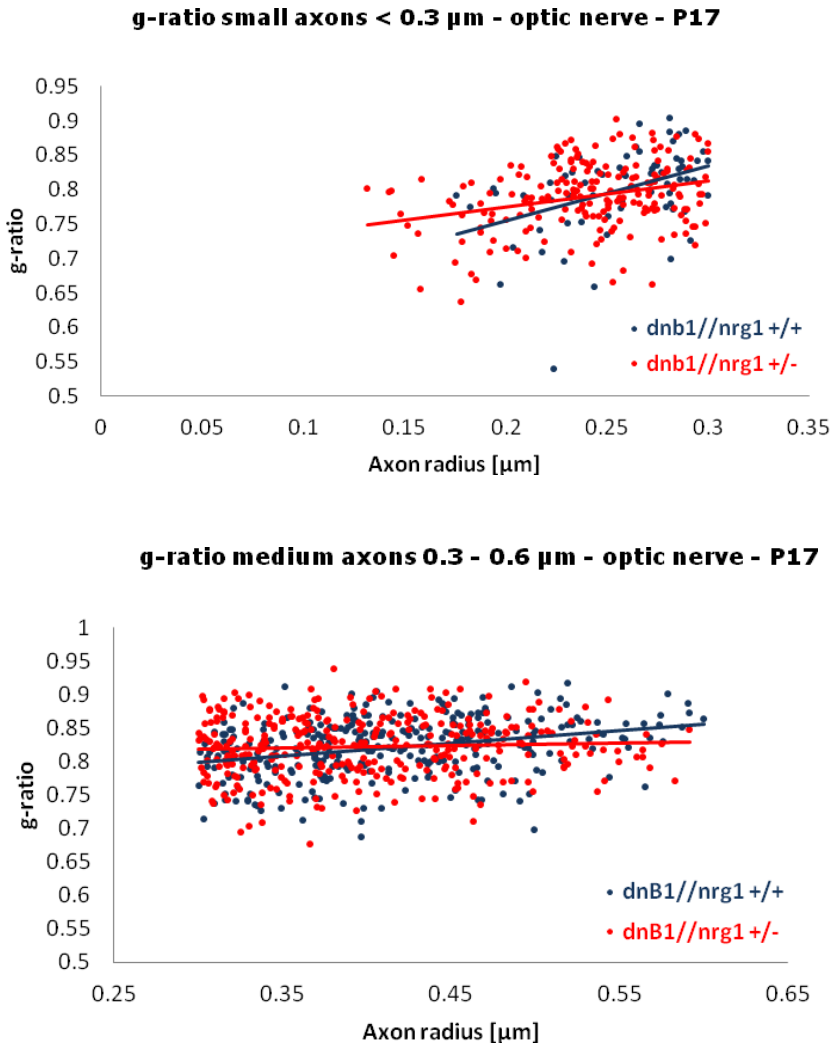


Figure 4.6 – Myelin thickness is unaltered in double mutant mice (dominant negative β1 integrin // neuregulin 1 type III heterozygous). Ration between the axon radius and axon radius plus myelin (g-ratio) plotted against the axons radius shows no difference (P=0.76) in myelin integrity between the dnβ1//nneuregulin 1 type III $^{+/+}$ (average g-ratio of 0.82 ± 0.00, n = 400 axons from 4 animals) and dnβ1//neuregulin 1 type III $^{+/-}$ mice (average g-ratio of 0.82 ± 0.014, n = 596 axons from 6 animals). Statistical analysis performed by Student's t test.

As previously seen in the dnβ1 mice, we saw no significant difference in the g-ratios of the dnβ1//neuregulin $1^{+/-}$ mice, showing that once oligodendrocytes establish contact with the axons, they are capable of correctly myelinating and forming

compact myelin. Since the previous phenotype described with the dnβ1 mice was detected in small diameter axons, we binned the data to assess whether there were any differences in the various axon radius categories (small < 0.3 µm; medium 0.3 – 0.6 µm, large > 0.6 µm). The binned data did not show any alteration of the myelin integrity (Fig. 4.7).



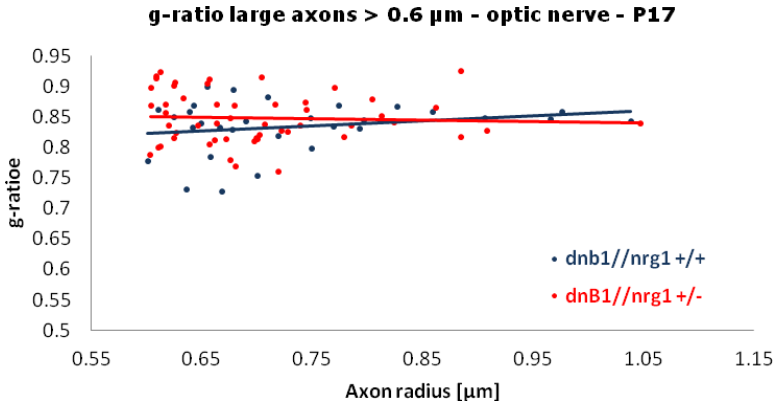


Figure 4.7 – Normal myelin thickness in the optic nerve of dominant negative β1 integrin // neuregulin 1 type III $^{+/-}$ mice. When the g-ratio is sub-divided according to various categories of axon diameter, no change is observed in myelin thickness in any particular group. G-ratio as a function of the different categories of axon diameter (small ≤ 0.3 µm, medium 0.3 – 0.6 µm and large ≥ 0.6 µm. dnβ1//nneuregulin 1 type III $^{+/+}$: n = 400 axons from 4 animals and dnβ1//neuregulin 1 type III $^{+/-}$ mice: n = 596 axons from 6 animals.

The dnβ1 mice showed a delay in the initiation of myelination of small diameter axons, when the percentage of myelination was analysed by plotting the axon diameter and the percentage of axons myelinated. To further assess if integrin β1 acts in concert with neuregulin 1 type III to regulate the initiation of myelination, we repeated the same type of analyses on the dnβ1//neuregulin 1 $^{+/-}$ mice and their dnβ1//neuregulin 1 $^{+/+}$ counterparts. A minimum of 400 axons were analysed for each animal, from at least 3 non-overlapping photos. These axons were classified as myelinated or unmyelinated and plotted by diameter. In the dnβ1 // neuregulin 1 $^{+/+}$, we have analysed 4 animals with an average percentage of myelination of 56.00 ± 3.305, and 6 animals were analysed for dnβ1 // neuregulin 1 $^{+/-}$ with an average of 56.00 ± 3.315 (P = 0.41). This shows that neuregulin 1 does not enhance the phenotype previously described for the dnβ1 integrin mouse (Fig. 4.8).

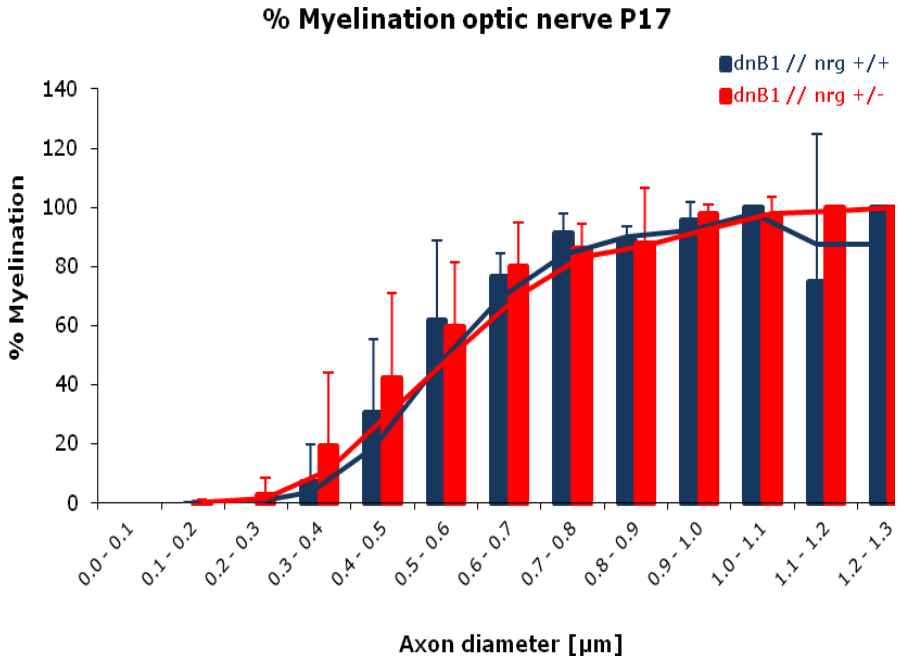


Figure 4.8 – Neuregulin 1 does not enhance the phenotype observed in the dominant negative β1 integrin. Percentage of myelinated axons plotted as function of the axon diameter shows no difference (P=0.41) between the dominant negative β1 integrin // neuregulin 1 type III $^{+/+}$ (with average of 56.00 ± 3.305, from 4 animals, n = 1780 axons) and the dominant negative β1 integrin // neuregulin 1 type III $^{+/-}$ (with average of 56.00 ± 3.315, from 6 animals, n = 2607 axons). These shows that neuregulin does not enhance the threshold for myelination observed in the dominant negative β1 integrin mice. A two-way ANOVA was performed for statistical analysis.

Since the phenotype observed in the dnβ1 is transient, with older mice not showing a delay in the myelination of small diameter axons, and to further test our hypothesis, we have analysed mice at postnatal day 21 (P21). A morphological analysis of the g-ratio in the dnβ1 // neuregulin $1^{+/+}$ and dnβ1 // neuregulin $1^{+/-}$ optic nerves of adult mice revealed no differences (P=0.22), with average g-ratios of 0.81 ± 0.007 (n = 592 axons, from 6 animals) and 0.82 ± 0.005 (n = 1301, from 13 animals) for the dnβ1 // neuregulin 1 $^{+/+}$ and dnβ1 // neuregulin 1 $^{+/-}$, respectively.

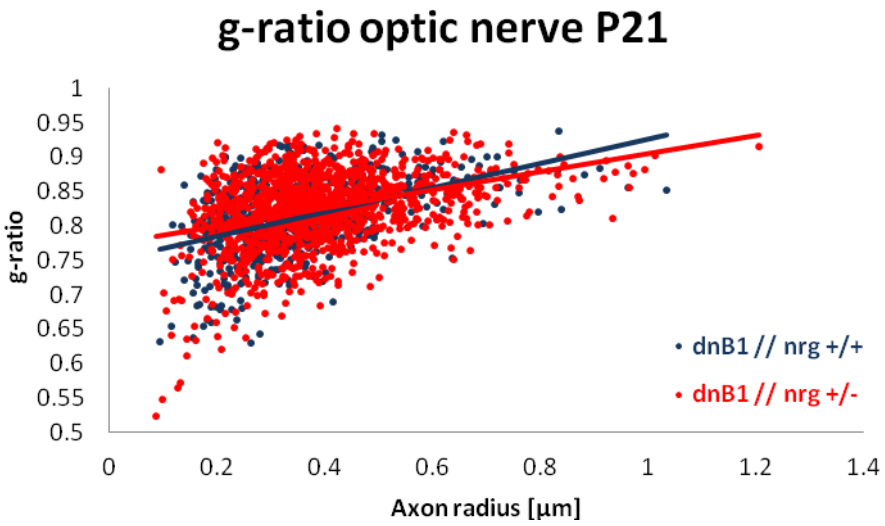
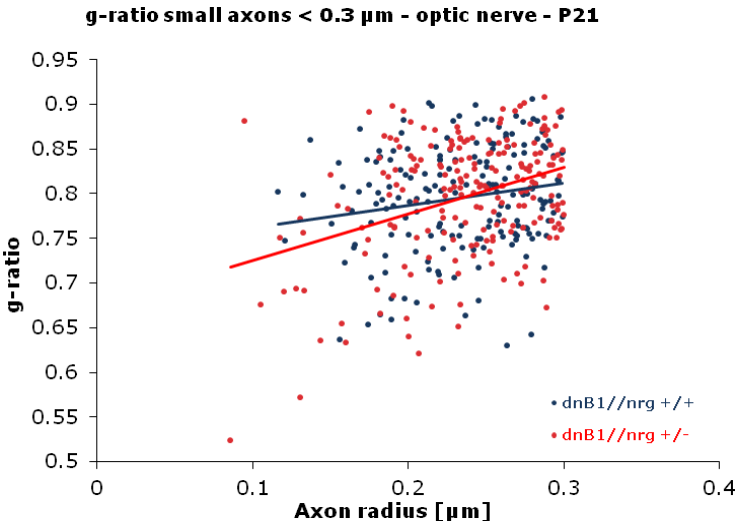


Figure 4.9 – Morphological analysis of the optic nerve at postnatal day 21 (P21). G-ratio measurements as a function of axon radius is not significantly different (P=0.22) between genotypes, with dnβ1 // nrg1 $^{+/+}$ having an average of 0.81 ± 0.007 (6 animals, n = 592 axons) and dnβ1 // nrg1 $^{+/-}$ (13 animals, n = 1301 axons) having an average of 0.82 ± 0.005. A Student's t test was used for statistical analysis.

We further grouped the axons by radius category and analysed the g-ratios for small (< 0.3 µm), medium (0.3 – 0.6 µm) and large (> 0.6 µm) radius. Consistent with the previous results, no difference was observed across the different classes of axon radius (Fig 4.10).



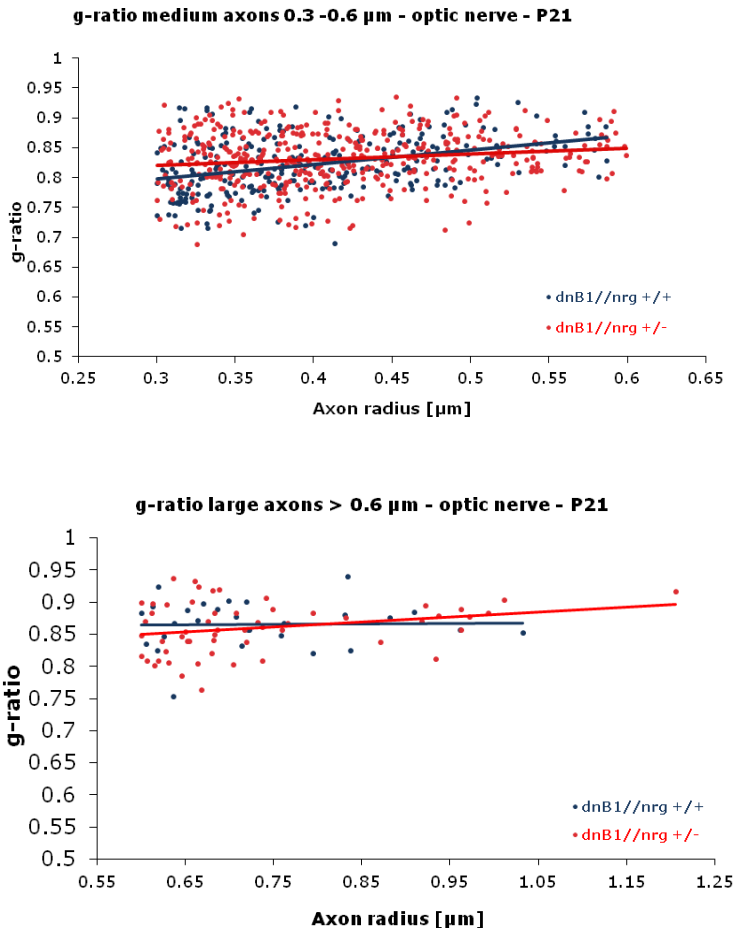


Figure 4.10 - Ultrastructural analysis of the optic nerve of dominant negative β1 integrin // neuregulin 1 type III $^{+/-}$ mice shows no abnormalities of myelination in different categories of axon diameter at postnatal day 21 (P21). G-ratio was divided according to different categories of axon diameter to confirm there was no specific phenotype in the different classes of axons. G-ratio measurements as a function of different diameters (small ≤ 0.3 µm, medium 0.3 – 0.6 µm and large ≥ 0.6 µm). dnβ1 // nrg1 $^{+/+}$: 6 animals, n = 592 axons and dnβ1 // nrg1 $^{+/-}$: 13 animals, n = 1301 axons.

To establish if the reduced levels of both integrin and neuregulin affect the threshold for myelination, we determined the percentage of myelination of the dnβ1 // neuregulin 1 $^{+/+}$ (average of 0.81 ± 0.007) and dnβ1 // neuregulin 1 $^{+/-}$ (average of 0.82 ± 0.005) mice, which showed no differences (P=0.21) (Fig 4.11).

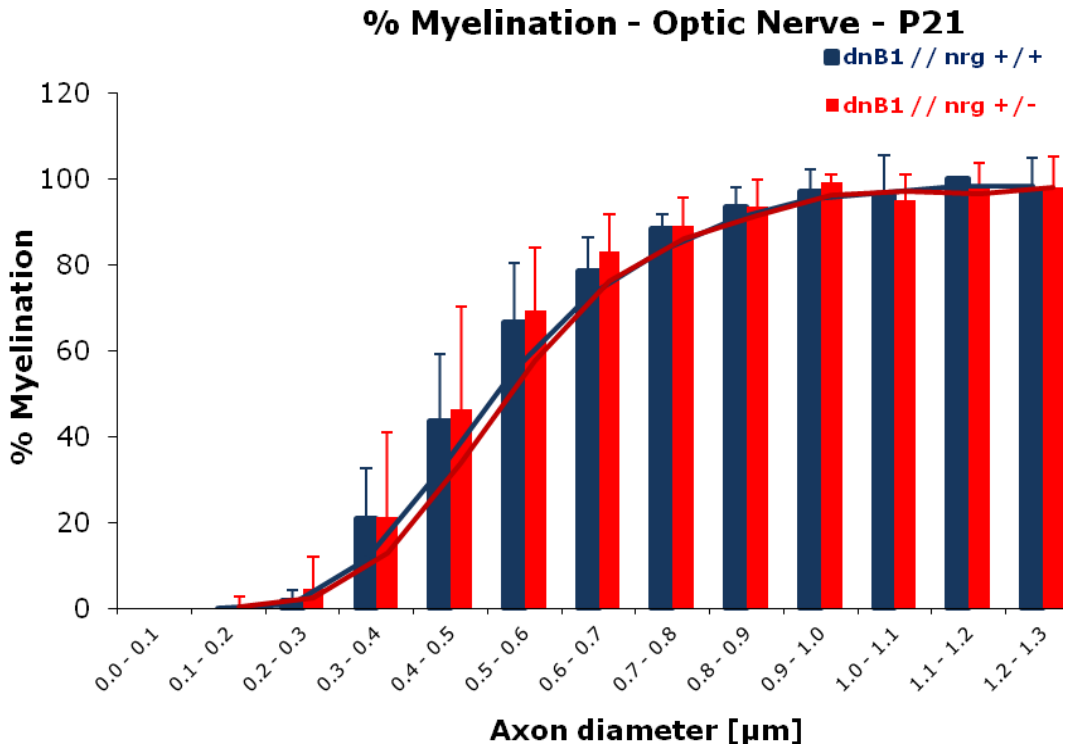
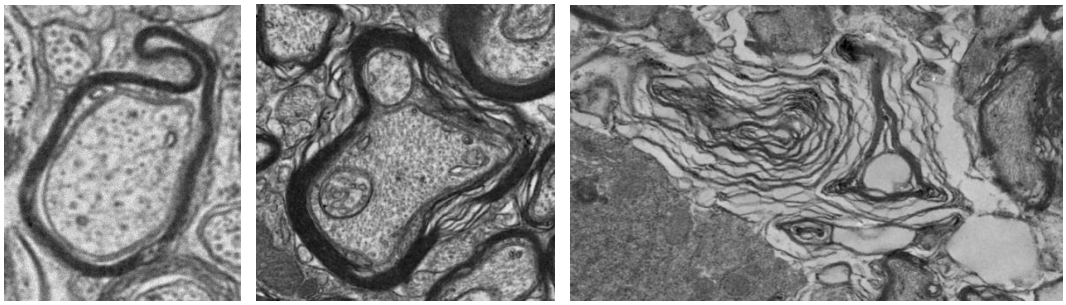
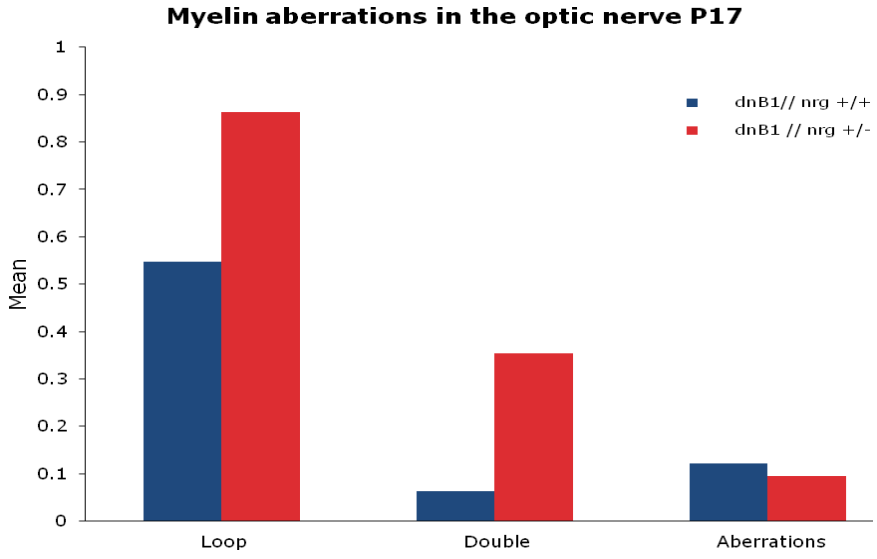


Figure 4.11 – The threshold for myelination is not increased in the optic nerve of dominant negative β1 integrin // neuregulin 1 type III $^{+/-}$ mice at postnatal day 21. The percentage of myelinated axons in plotted against axon diameter at 0.1 intervals shows no difference in between genotypes. dnβ1 // neuregulin 1 $^{+/+}$ mice show an average of percentage of myelinated axons of 0.81 ± 0.007, from 6 animals, n = 2510 axons) and dnβ1 // neuregulin 1 $^{+/-}$ mice had an average of 0.82 ± 0.005, from 13 animals (n = 5451 axons), with a *p* value of 0.21. A two-way ANOVA was performed for statistical analysis.

While analysing the data, it was apparent that these animals presented different types of myelin aberrations. Therefore we categorized them as **a)** loops, where myelinated axon(s) are wrapped with a loop of uncompacted myelin; **b)** double wraps, where more than one axon is wrapped in the same myelin sheath; and **c)** dystrophies, where either there is a spiral of uncompacted myelin due to axon degeneration, or loose myelin surrounding multiple axons. After quantifying each category, we found that there was no significant difference between any of the categories at the different time points.





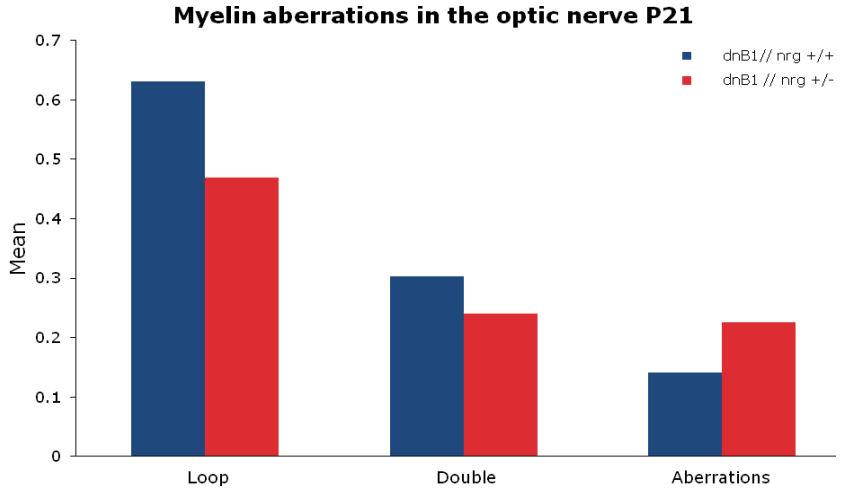


Figure 4.12 – Quantification of myelin aberrations in the optic nerve of dominant negative β1 integrin // neuregulin 1 type III +/- mice. Upper panel: representative electron

micrographs of the different categories of myelin aberrations: loop, double wrap and dysmyelination. Mean for dominant negative β1 integrin // neuregulin 1 type III +/+ mice: loop (0.55), double (0.06) and aberrations (0.12) (from 4 animals). Mean for dominant negative β1 integrin // neuregulin 1 type III +/- mice: loop (0.86), double (0.35) and aberrations (0.09) (from 6 animals). Middle panel: quantification of myelin aberrations in the optic nerve at postnatal day 17 (P17). Bottom panel: myelin aberrations in the optic nerve of P21 mice. Mean for dominant negative β1 integrin // neuregulin 1 type III +/+ mice: loop (0.63), double (0.30) and aberrations (0.14) (from 6 animals). Mean for dominant negative β1 integrin // neuregulin 1 type III +/- mice: loop (0.47), double (0.24) and aberrations (0.23) (from 13 animals). This analysis was performed by simply counting the number of myelin aberrations for each sample, from at least 5 non-overlapping electron microscopy images. A Wilcoxon signed-rank test was performed for statistical analysis, this test is performed by hand and showed there was no significant difference between both phenotypes, in the different categories of myelin aberrations. According to the Wilcoxon signed-rank test a rank of the differences between each pair of scores was paired and ranked. A value of the differences between the ranks (W) is then compared with the table of critical Wilcoxon values. Since this value was higher than the table of critical values for the Wilcoxon test, no statistical difference is present in these samples.

Initially, neuregulin 1 type III $^{+/-}$ mice were reported to be hypomyelinated in the corpus callosum (Taveggia *et al.*, 2008). To investigate if the added disruption of β1 integrin signalling would extend these observations, we analysed the morphology of myelin thickness by g-ratio at postnatal day 17 and 21 (Fig 4.13 and 4.14). At P17, analysis of 3 animals per genotype showed no differences (P=0.40) between the dnβ1 // nrg1 $^{+/+}$ (average of 0.73 ± 0.02, n = 321 axons) and dnβ1 // nrg1 $^{+/-}$ (average of 0.77 ± 0.04, n = 303 axons). Myelin thickness also showed a trend of being unaltered in the P21 mice with averages of 0.72 (n=207 axons, from 2 animals) and 0.73 (n=206 axons, from 2 animals).

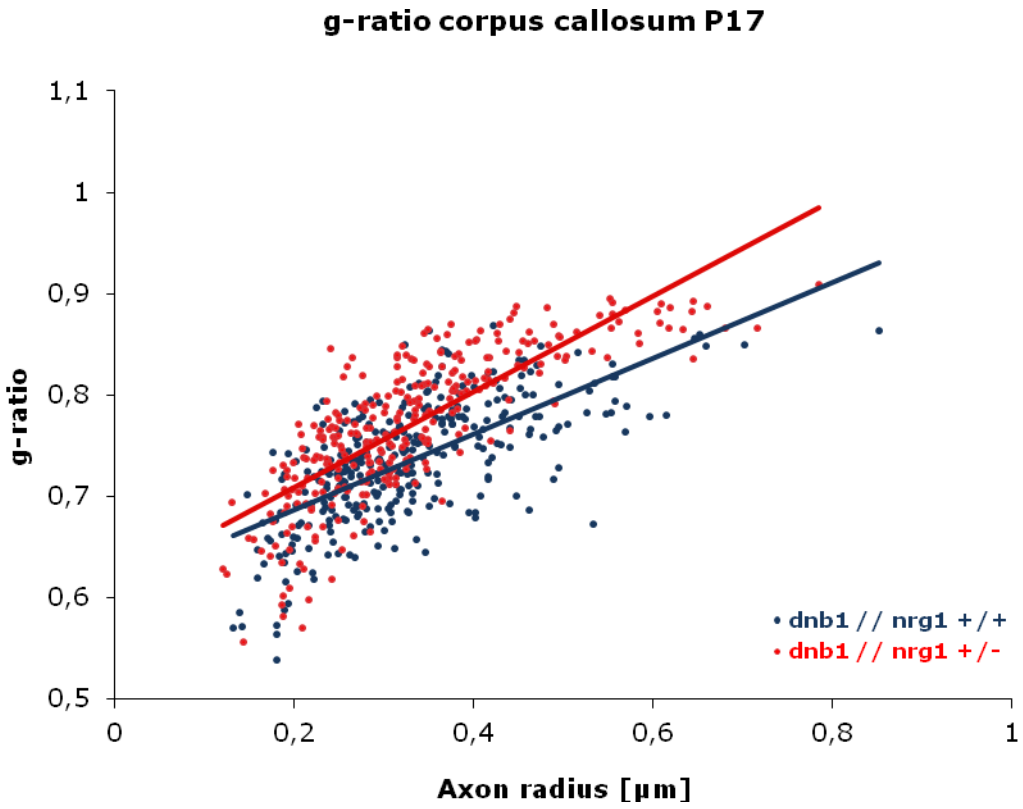


Figure 4.13 – β1 integrin // neuregulin 1 type III signaling does not affect myelin thickness in the corpus callosum *in vivo* at postnatal day 17 (P17). No significant difference (P = 0.40) was observed between genotypes, showing a normal myelin integrity between the dnβ1 // neuregulin 1 $^{+/+}$ (3 animals; average of 0.73 ± 0.02, n = 321 axons) and dnβ1 // neuregulin 1 $^{+/-}$ (3 animals; average of 0.77 ± 0.04, n = 303 axons). Statistical analysis performed by Student's t test.

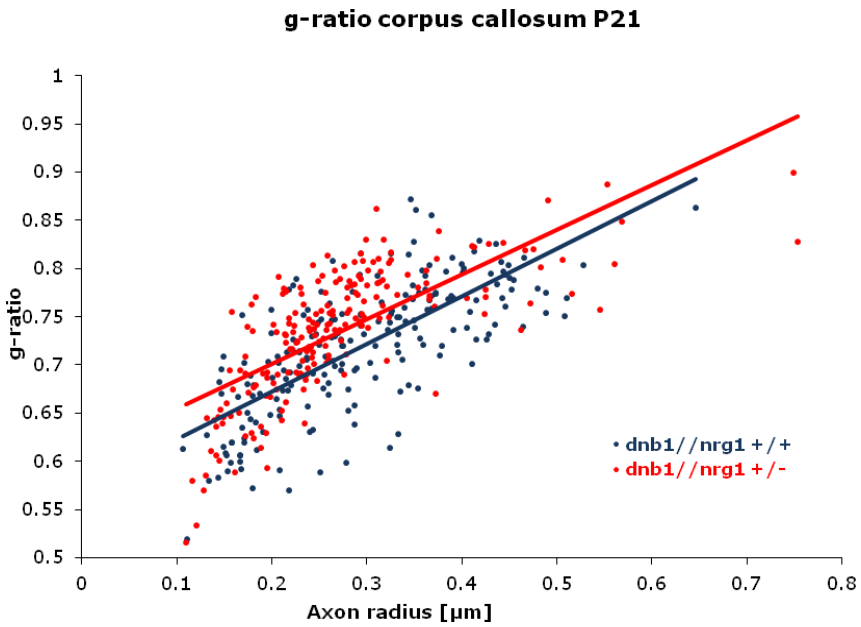


Figure 4.14 – Unaltered myelin morphology in the corpus callosum of dominant negative β1 integrin // neuregulin 1 type III heterozygous mice at postnatal day 21 (P21). Disruption of integrin and neuregulin signaling results in normal myelin thickness in the corpus callosum of dnβ1 // neuregulin 1 $^{+/-}$ mice (2 animals, average of 0.73; n=206 axons) compared with dnβ1 // neuregulin 1 $^{+/+}$ (2 animals, average of 0.72; n=207 axons.

Taken these results together, we conclude that neuregulin 1 type III does not enhance the β1 integrin phenotype described in the dominant negative β1 mice.

4.3 Discussion

Myelination is a key feature for all organisms allowing the nervous impulse to propagate faster along the axon. In diseases such as Multiple Sclerosis (MS) where new myelination (remyelination) is impaired, it is key to understand the mechanisms that control myelination in order to develop drug targets used to promote remyelination. In MS lesions, oligodendrocytes are arrested in a premyelinating state (Kuhlmann *et al.*, 2008). The ability of oligodendrocytes to extend multiple processes and myelinate different internodes points out the complexity of the myelination process, where oligodendrocytes must initiate axoglial contact, sense the axon size and wrap the necessary number of myelin sheaths to efficiently promote

insulation of the axon. Throughout the years researchers have tried to identify the molecules that regulate myelination. Work described in this thesis (Chapter 3) showed that one of these molecules is β1 integrin. Dominant negative β1 integrin mice showed that β1 integrin signalling regulates the initial stage of myelination with mutant mice presenting a delay in myelination compared to the wildtype. This effect is specific for small diameter axons and suggests that mutant oligodendrocytes are unable to recognize or sense these small calibre nerves and initiate myelination. Interestingly, the alteration described in myelination is transient with older mice showing that oligodendrocytes eventually recover their ability to successfully myelinate small calibre fibbers. Neuregulin 1 type III is expressed on the axonal surface and in the PNS (where Schwann cells – cells responsible for myelination – establish axoglial contact with only one axon) has been identified as a key axonal signalling molecule that regulates myelin thickness and glial fate.

In the PNS, neuregulin I type III has been identified as a necessary and sufficient signal that regulates axoglial interaction. The levels of axonal Nrg1 regulate whether an axon is myelinated and also controls myelin thickness. Overexpression of neuregulin 1 type III on normally unmyelinated axons promotes their myelination *in vivo*, while transgenic mice expressing higher than normal levels on peripheral nerve axons generate thicker myelin sheaths (Michailov *et al.*, 2004; Taveggia *et al.*, 2005).

In the CNS, the action of neuregulin remains unknown with conflicting reports showing that neuregulin 1 can regulate myelination *in vitro* (Taveggia *et al.*, 2008; Wang *et al.*, 2007) and *in vivo*. Mice haploinsufficient for neuregulin 1 type III show hypomyelination of the corpus callosum, but no defects in the optic nerve and spinal cord (Taveggia *et al.*, 2008). Contrasting with these reports, Brickmann and co-workers described no alterations of cortical myelination in mice with ablated neuregulin (Brickmann *et al.*, 2008).

As mentioned above, our dominant negative β1 integrin mice presented a transient effect showing that disruption of β1 integrin signalling is either only necessary for the initial stages of myelination or that compensatory mechanisms act to restore

normal myelination. Given the evidence from the PNS, we hypothesised that integrin and neuregulins act in concert and form a signalling complex responsible for initiation of myelination. If this was the case, disruption of both signalling molecules would have a greater effect than the one seen in the dnβ1 and the neuregulin 1 type III $^{+/-}$ mutant mice alone. To test our hypothesis, we crossed these mice and predicted an enhanced phenotype to the one described in the optic nerve of the dnβ1 and further hypomyelination of the corpus callosum. By analysing the optic nerve and corpus callosum of the offspring of this cross at postnatal day 17 and 21, we conclude that this is not the case. In neither the structures analysed was the morphology of the myelin sheath or its thickness altered, and percentage of myelinated axons did not intensify the shift to the right of the dose-response curve initially observed in the dnβ1.

From previous studies in the PNS it is clear that neuregulin 1 type III controls the Schwann cell differentiation mechanism and myelination (Garrat *et al.*, 2000; Jessen and Mirsky, 2005; Nave and Salzer, 2006). Given the similarities in the myelin sheath composition of the PNS and CNS, it would be expected that Nrg1 had an analogous function in central myelination. This would make neuregulin 1 a particularly good candidate for clinical applications, both in multiple sclerosis and also in schizophrenia. Work described in this chapter together with the results of Brickmann and colleagues (2008) points to different molecular factors that contribute to PNS and CNS myelination, with neuregulin 1 being redundant for central myelination. Both these results are in conflict with other *in vivo* and *ex vivo* data. It is difficult to reconcile both *in vivo* studies, since neuregulin 1 type III happloinsufficient mice present hypomyelination of the corpus callosum (Taveggia *et al.*, 2008). One possible explanation would be the background of the strains used in both studies. *Ex vivo* data, where spinal cord explants from mice with ablated neuregulin 1 and ErbB2 were cultured showed that oligodendrocytes are unable to differentiate (Park *et al.*, 2001; Vartanian *et al.*, 1999). Brickmann proposed that the lack of electrical activity in these cultures could force myelination into a parallel and simplified mechanism of myelination (Brickmann *et al.*, 2008).

It is important to note the complexity of central myelination, when compared to PNS. Oligodendrocytes can myelinate up to 40 internodes, therefore they need to extend several processes that would have to be tightly regulated. While Schwann cells establish a 1:1 ratio with the respective internode of interest, these cells would, in theory, require just one transcription machinery, in contrast to oligodendrocytes that would require further post-transcriptional modification. It is also important to note that possible compensatory mechanisms, either by other neuregulins (such as neuregulin 2 or neuregulin 3) or neuregulin isoforms, could explain this lack of phenotype. It has been described that neuregulin 2 is structurally similar to neuregulin 1 type III and is expressed by DRGs (Carraway *et al.,* 1997). Although neuregulin 3 is not structurally similar to neuregulin 1 type III, both neuregulin 2 and neuregulin 3 transgenic mice show normal myelination (Zhang *et al.,* 1997). Although PNS and CNS share some features between them, the possible rationale to support these studies would be that PNS and CNS myelination diverged during evolution and Schwann cells retained simpler and ancestral regulatory mechanism for myelination, whilst oligodendrocytes developed a highly intricate network of parallel and compensatory pathways to protect CNS integrity.

Chapter 5

Neuregulin 1 and schizophrenia

Chapter 5

5.1 Introduction

Given the interest in neuregulin 1, next we decided to look at schizophrenia, a disease that has been linked with neuregulin 1 as one of its genetic risk factors.

Central hypothesis

Neuregulin 1 type IV overexpressing mice show a behavioural phenotype and reduced fractional anisotropy and white matter density in the corpus callosum compared to wild type mice. Therefore we hypothesised that these could be due to a myelination defect.

Statement of aims

1. To characterize myelination in the neuregulin 1 type IV overexpressing mice at different time points by analysing the g-ration through electron microscopy.
2. To test if mice overexpressing neuregulin 1 type IV show changes in myelin staining using immunohistochemistry at postnatal day 17 and 60.

Etymology of schizophrenia

Schizophrenia is one of the most common psychiatric illnesses. It is a chronic and debilitating disorder characterized by a triad of positive (e.g. delusions, hallucinations), negative (e.g. apathy, social withdraw) and cognitive (e.g. attention, working memory) symptoms (Rico and Marín, 2011). Historically, schizophrenia has long been reported (Jeste *et al.*, 1985), with Emil Kraepelin first describing it as "dementia praecox" (dementia of the young) based on the young age of patients and a chronic dementia. Although the typical age of onset is around 20 to 25 years of age,

this varies among patients, together with the fact that not all patients will develop dementia means that Kraepelin's definition was misleading (DeLisi, 1992). The term schizophrenia was later coined by Eugen Bleuer and etymologically means "split personality" and defined by "ambivalence, disturbance of association and affect, and a preference for fantasy over reality" (reviewed by Walker *et al.,* 2004). Once thought to be a neurodegenerative disease, it is now clear that schizophrenia is instead a neurodevelopmental disease since patients do not present neurodegeneration and present symptoms associated with schizophrenia (i.e. cognitive and social impairment) before onset of psychosis (Lewis and Lieberman, 2000).

Etiology of schizophrenia

Due to the multifactorial aspects surrounding schizophrenia the exact etiology of this disorder remains elusive. Apart from the different susceptibility genes involved with schizophrenia (such as neuregulin and DISC1) and environmental factors that are linked with neurodevelopment (such as pregnancy related, child and adult risk factors), and drug use, there are different theories about the neuropathological changes observed in schizophrenic patients which include various brain regions and the connection between them (Andreasen, 1996; Pulver, 2000; Tsuang *et al*., 2001; Weinberger, 1995).

One of the current models to explain the symptomatology of schizophrenia is the dopamine hypothesis, where dysregulation of dopamine in the mesolimbic pathways leads to the positive symptoms, while negative symptoms and cognitive impairments are associated with disruptive mesocortical dopamine pathways (Bensherif *et al*., 2012). This hypothesis is based on the altered dopamine D_2 receptor levels observed in post-mortem schizophrenic brains, as well as the levels of dopamine occupancy of these receptors, the induction of psychosis by dopamine agonists – such as amphetamine – in both humans and animal models, as well as the pharmacological action of neuroleptic drugs – that act as antagonist of the dopamine receptors (Ross *et al*., 2006). Although this is the most accepted hypotheses of the etiology of schizophrenia, the dopamine hypothesis does not explain all the underlying

symptoms of schizophrenia. The current knowledge of the interaction of different neurotransmitters in the brain has led to an increasing interest on their role in schizophrenia, suggesting that different neurochemical systems might be involved in this pathology. Since most antipsychotics target the dopamine receptor, it would be expected (based on the dopamine hypothesis) that these would be an effective treatment, unfortunately (with exception of clozapine) most neuroleptic drugs only affect positive symptoms.

One of the complementary hypotheses involves glutamate, a neurotransmitter mediated by three different types of receptors, AMPA, kainite and NMDA. The NMDA receptor hypofunction hypothesis is based on the evidence that NMDA receptor antagonists (eg, phencyclidine – PCP, ketamine and MK-801) can mimic a different range of psychotic features of schizophrenia in normal individuals, and these are reproducible in experimental animals (reviewed by Coyle, 2012). The disruptions generated by the NMDA receptor antagonists resemble some of the negative and neurocognitive symptoms associated with schizophrenia.

Although the dopamine and glutamate hypothesis shed light into the positive and negative symptoms associated with schizophrenia, patients also present cognitive deficits that do not seem to be fully regulated by either neurotransmitter system. A decreased level of gamma-aminobutyric (GABA) neurotransmitter and GABA transporter is thought to underlie the cognitive impairments of schizophrenia, since both have been observed in schizophrenic patients (Yoon *et al*., 2010). The GABAergic system has a role in the excitation/inhibition balance in the brain and is ubiquitously present in the CNS, therefore one of the hypotheses is that GABA, which can regulate the glutamate-induced excitation, is that a disruptive interaction between GABA and the glutamate and/or dopamine systems is involved in the cognitive deficits observed in schizophrenia (Costa, 1992).

Diagnosis of schizophrenia

The most widely used criteria for the diagnostic of schizophrenic patients is based in the America's Association Diagnostic and Statistical Manual of Mental Disorders (version DSM-5) (American Psychiatric Association, 2013). According to DSM-5 a patient has to present signs and symptoms for six months or longer. At least two or more of the following symptoms should be present for at least a month: hallucinations, delusions, disorganized speech, grossly disorganized behaviour or catatonic behaviour, and negative symptoms such as lack or decline of emotional response, lack or declined in speech and lack or decline in motivation (American Psychiatric Association, 2013). Although these diagnostic guidelines are currently in use it is to note that diagnosing patients with schizophrenia is difficult since some symptoms might be due to other mood disorders such as bipolar disease (Kendell, 1975). Currently, a patient can only be diagnosed based on the clinical features and medical assessment, but this can prove a difficult task due to heterogeneity of schizophrenia and overlapping symptomatology with other psychiatric disorders. Biomarkers that can be used as a diagnostic tool in schizophrenia have long been sought after (Takesada *et al*., 1965). Substantial research has been done since, but thus far no biomarker (either for diagnosis, prognosis or a theranostic biomarker) for clinical use has been documented (reviewed by Weickert *et al*., 2013).

Treatment of schizophrenia

Schizophrenia has no known cure. All treatments available target specific symptoms associated with the disorder, with long term treatment available for management of positive symptoms, and some negative symptoms. The cognitive deficits associated with schizophrenia have a modest response to the current available treatments (Furth *et al.,* 2013). Current treatment consists of typical and atypical antipsychotics. Typical antipsychotics (also known as first generation antipsychotics) such as chlorprozamine, fluphenazine and perphenazine, have a long history of use, being introduced for the treatment of schizophrenia in the 1950s. Their mechanism of action is associated with a high affinity for dopamine D_2-receptors that regulate the

release of dopamine and this characteristic can lead to severe extrapyramidal side effects such as tardive dyskinesia, as a result of hypodopaminergic transmission. Atypical antipsychotics (or second generation antipsychotics) such as clozapine, quetiapine and ziprasidone were introduced in the last three decades and share a similar pharmacological mechanism as their typical counterparts but have a lower risk of extrapyramidal side effects, and have therefore become the first line prescription. The contrast in terms of extrapyramidal side effects is thought to be due to differential effects of typical and atypical antipsychotics on the striatal striosome and matrix compartments (Bubser and Deutch, 2002).

Treatment for schizophrenia has had a few major innovations in the last decade, but due to the highly polygenetic nature of schizophrenia, new therapies should be based on genetic and epigenetic studies in order to provide a full understanding of the neuropathology of schizophrenia and a more adequate and targeted therapy (Marden *et al*., 2011).

5.1.1 Risk factors of Schizophrenia

Schizophrenia underlies a combination of environmental and genetic factors that are thought to contribute to a disturbed neurodevelopment, but a single (and exclusive) factor that regulates the pathophysiology of schizophrenia has not been identified. Epidemiological studies show that 1% of the population worldwide is affect by schizophrenia and studies point out for an estimated heritability between 66% and 80% (Cardno *et al.,* 1999; Owen *et al.,* 2005).

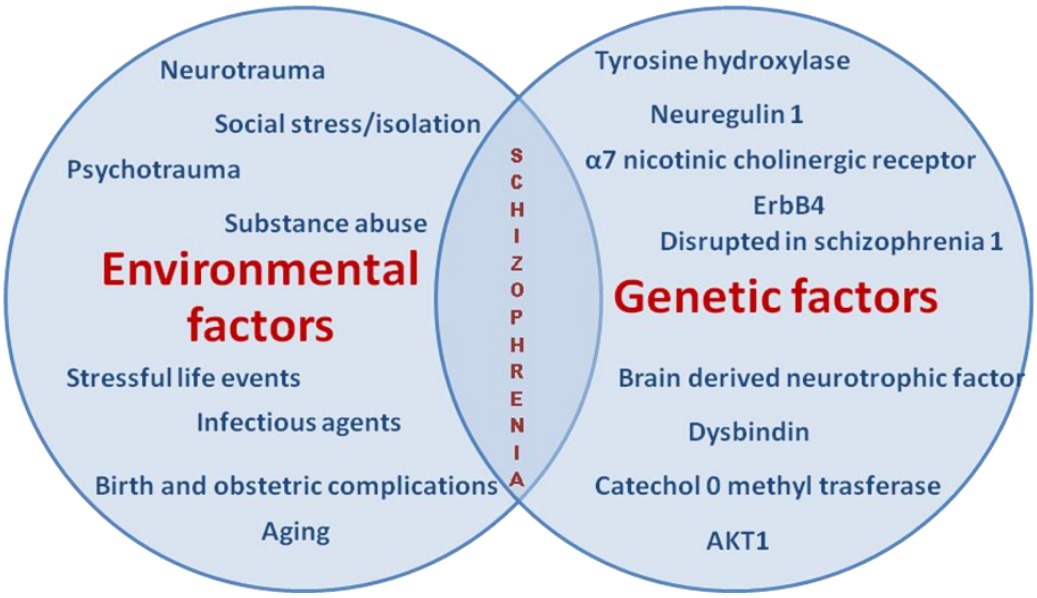


Figure 5.1 – Confounding factors of schizophrenia. Schizophrenia is a multifactorial psychiatric disorder with multiple genetic and environmental associated risk factors. In individuals with a genetic load of susceptibility to environmental factors, a potential cofactor - such as perinatal trauma - can lead to spontaneous schizophrenia. In some cases, even if the individual does not carry a susceptibility gene a neurochemical disbalance caused by external factors such as substance abuse, neurotrauma or even a stressful life event can precipitate an atypical schizophrenic psychosis.

Environmental factors

Schizophrenia is a complex multigenetic disease. Multiple genetic risk factors have been identified (such as neuregulin and DISC1), but the onset of the disease is also dependent on environmental factors. A potential cofactor such as perinatal trauma can lead to spontaneous schizophrenia in individuals with a genetic predisposition. Additionally, a disbalance of external factors such as substance abuse, infectious agents, aging or psychotrauma can lead to a late onset of schizophrenia, without a genetic predisposition.

Epidemiological studies have suggested that prenatal infection and the successive activation of the maternal immune system is linked with the risk of developing schizophrenia later in life (Canetta and Brown, 2012). Most of the studies associating prenatal infection with the risk of schizophrenia (and psychosis) were based on cohorts studies of maternal infection based on serology or medical records (reviewed by Brown and Derkits, 2010). These epidemiological studies combined with human, preclinical and animal studies have unveiled the morphological, molecular and neurochemical alterations present in both schizophrenic patients and animal models of prenatal immune activation. Neurochemical features show alterations in neurotransmitters such as dopamine, serotonin and glutamate. Consistent with reports from schizophrenic patient, animal models show reduced white matter volume and hippocampal neurogenesis and raised microglial activation. Other environmental factor that have been associated with schizophrenia consist of obstetric complications, that comprise both birth and pregnancy complications. Different cohort studies have shown that intrauterine growth retardation, preterm births, perinatal trauma (such as brain injuries or hypoxia-ischemia), low birth weight or use of resuscitation or incubator increases the risk of schizophrenia (Geddes *et al.,* 1999; Hultman *et al.,* 1999; Jones *et al*., 1998). Remarkably, month of birth is also a cofounding factor in schizophrenia with late winter/early spring births having a higher risk of developing schizophrenia, thought to be related to maternal infection (since the births succeed the typical months for influenza infection) that may lead to abnormal brain development (reviewed by McDonald and Murray, 1999).

Although the genetic and environmental factors are the core features of schizophrenia, it is interesting that the onset of the disease is predominantly during adolescence since most of these factors are present since birth. Most individuals that develop schizophrenia show some mild debilitating characteristics early on, either cognitive (with learning difficulties or low IQ) or social (Done *et al.,* 1994; Foerster *et al*., 1991), demonstrating that some of the traits of the disease are present before onset. One of the explanations proposed is the theory of "doomed from the womb", although the genetic and environmental predisposition are present from early life, and some traits are shown since childhood these might be due to abnormal neuronal

adaptation and maturation that will subsequently impair neuronal networks and promote the development of psychosis (reviewed by McDonald and Murray, 1999).

Drug abuse and traumatic life events are also thought to be risk factors for developing schizophrenia, usually with a later onset. Substance abuse (especially cannabis) can lead to psychotic episodes that can develop into schizophrenia, with a correlation between the amounts of cannabis used and development of schizophrenia (Andreasson *et al.,* 1987). Cannabis, specifically its main component THC, interacts with the dopaminergic system raising dopamine in the brain and therefore increases the risk of onset of psychosis (Tanda *et al.,* 1997).

Genetic vulnerability

Schizophrenia is a complex disease, with multiple genetic, environmental, and stochastic factors that can act as cofactors. It is therefore important to identify the genetic basis of schizophrenia for a better understanding of the etiopathogenesis of this disorder, as this will grant a better knowledge for the development of targeted therapies. It is thought that genetic factors contribute to 50% of the risk of developing schizophrenia (Siegel and Ralph, 2011). So far, 15 possible genetic loci linked with schizophrenia have been identified mainly through linkage and association studies from family, twins and adoption cases (Marden *et al*., 2011).

One of the candidate genes for risk of susceptibility in schizophrenia (disrupted in schizophrenia 1 - Disc1) was identified in a Scottish family with high prevalence of mental illness such as schizophrenia, bipolar affective disorder and major depression (Millar *et al*., 2000) and these findings have been corroborated by post-mortem, genetic and animal studies (reviewed by Hennah *et al.,* 2006; Porteous and Millar., 2006). This gene is expressed in humans, rodents and non-human primates and it is expressed both in neurons and glial cells, reaching its peak levels during neurogenesis and adolescence (Brauns *et al.,* 2011). Research has undercover a role for Disc1 in several aspects of mental illness such as working memory deficits, a decrease in volume and density of grey matter, impaired function and volume of the

hippocampus, and interestingly psychosis-related traits in healthy controls (Cannon *et al.*, 2005; Hashimoto *et al.,* 2006, Hennah *et al.,* 2005, Tomppo *et al.*, 2009). Disc1 has been implicated in an array of biological processes regarding development and maturation such as extracellular signalling, neurogenesis, cytoskeletal modulation, neurite proliferation, migration and outgrowth, synapse formation and maturation, decreased brain volume and altered behavioural phenotypes resembling schizophrenia and major depression in mutant mice (Clapcote *et al*., 2007; Lee *et al*., 2011; Morris *et al*., 2003), To further investigate the role of Disc1 diverse mutant mice lines have been established, providing insight into the biological and behavioural role of Disc1. Interestingly, Clapcote and co-workers generated two different mutations on exon 2 of Disc1 gene with different outcomes. Mutation Q31L showed a behavioural phenotype that resembles depression, while the L100P mutation resulted in a schizophrenia-like phenotype, demonstrating that these two pathologies have a common genetic factor (Clapcote *et al.,* 2007). Transgenic mice for Disc1 also present an increased level of dopamine D_2 receptors in the striatum, a brain structure highly implicated in mental illness, and these results were also corroborated in human patients (Chakracarty *et al.*, 2012). In humans, there has been extensive screening of patient's cohorts and linkage studies for Disc1 mutations all demonstrating a correlation between Disc1 and mental illness (reviewed by Porteous *et al.,* 2006). Human research has showed that individuals affected by mental illness and carrying a DISC1 mutation present a decreased volume of the prefrontal cortex and left supramarginal gyrus, consistent with the typical MRI scans of schizophrenic patients (Brauns *et al.*, 2011; Szeszko *et al*., 2008),

Neuregulin 1 was originally associated as a susceptibility gene for schizophrenia in studies of families in Iceland that identified an encoding region for neuregulin 1 (Stefansson *et al.,* 2002). Epidemiology studies suggest a link between several mutations of the neuregulin 1 gene (around 80 single-nucleotide polymorphisms (SNPs) have been identified so far) and schizophrenia in distinct populations' worldwide (reviewed by Mei and Xiong, 2008). Although neuregulin 1 is a candidate gene for schizophrenia-associated mutations a correlation between neuregulin 1 and schizophrenia was not observed in specific populations such as Japanese, Irish and

Spanish (Iwata *et al.,* 2004; Rosa *et al.,* 2007; Thiselton *et al.,* 2004). This suggests that neuregulin 1 can influence the onset of schizophrenia but other factors/genes are involved in this pathology.

Other genes have been implicated along with neuregulin and Disc1, are dysbindin, which in healthy individuals is associated with working memory, but in schizophrenic patients, which have a reduced expression level of dysbindin in the hippocampus and prefrontal cortex, results in a decreased excitatory cortical limbic synapses that contributes to the cognitive deficits seen in these subjects (Carlson *et al.,* 2011; Gill *et al.,* 2010; Jaaro-Peled *et al.,* 2009); and Neurexin-1, which has also been implicated in autistic spectrum disorder, plays a role in the formation and maintenance of synapse. In schizophrenic patients two of the proteins encoded by the neurexin-1 gene are affected differently, with no changes in expression in neurexin-1β and a deletion of the neurexin-1α promoter leading to an absence of neurexin-1α in these subjects (Zweir *et al.,* 2009). Transgenic mice lacking neurexin-1α expression show a decreased spontaneous excitatory synaptic transmission and in behavioural tests present a decrease in prepulse inhibition (PPI), a measurement of sensory gating that is also observed in schizophrenic patients (Etherton *et al.,* 2009). This is not a comprehensive list of genes that have been associated with schizophrenia, and for the context of this thesis the focus will be on Nrg1/ErbB role in schizophrenia.

As a future research target, further studies onto these interchangeable cofactors (environmental and genetic) should be carried out in order to address whether solely environmental factors can lead to schizophrenia, or if a genetic coadjuvant needs to be present, and if this is the case, if there is a specific relationship between distinct risk factors.

5.1.2 Neuregulin 1 and ErbB signalling in Schizophrenia

The features of the symptoms of schizophrenic patients, together with a known neurodevelopment pathophysiology indicates that there is a genetic predisposition, in addition to environmental factors, involved in this psychiatric disorder. Recently, gene expression studies have found various genes in the prefrontal cortex of patients with mental disorders, including schizophrenia, that have an altered expression and are involved in myelination, such as neuregulin 1. Neuregulin 1 (Nrg1) is the most well studied member of the neuregulin family (comprising neuregulin 1-4) and belongs to the epidermal growth factor (EGF) superfamily. *NRG1* gene encodes different isoforms through alternative splicing and differential promoter usage that have been previously described in Chapter 4. In recent years the human specific type IV isoform (specifically the [T/T] allele) has been associated with schizophrenia.

Neuregulin 1 type IV levels are higher in schizophrenia patients when compared to control individuals (Law *et al.,* 2006). Initial demonstration of association between Nrg1/ErbB signalling and schizophrenia was provided by studies of gene expression profile in the prefrontal cortex of schizophrenic patients that showed decreased levels of ErbB3, leading to a decreased expression of oligodendrocyte-specific genes (Hakak *et al.,* 2001). *In vivo*, ablation of one copy of the Nrg1 gene, results in abnormal behavioural phenotype in mice similar to schizophrenic symptoms, such as hyperactivity and decreased prepulse inhibition. These are reversible with anti-psychotic drugs (Stefansson *et al.,* 2002). To further deepen the knowledge of the relationship of neuregulin 1 and environmental factors, Desbonnet and colleagues analysed transgenic mice (heterozygous for neuregulin 1) with another cofounding factor – chronic social stress (Desbonnet *et al*., 2012). Mice with heterozygous deletion of the neuregulin 1 transmembrane display a range of behavioural deficits consistent with schizophrenia such as an increased exploratory behaviour when in a new environment (O'Tuathaigh *et al*., 2010), in contrast animals exposed to social defeat showed anhedonia, and a decrease level of explorative behaviour. When the neuregulin 1 transgenic mice were exposed to social defeat, the initial exploratory

effect was absent, leading to the conclusion that environmental factors can influence genetic behavioural outcomes (Desbonnet *et al*., 2012).

Several functions have been attributed to the Nrg1/ErbB signalling in the nervous system such as radial neuron migration, axon guidance, oligodendrocyte and Schwann cell development (previously described in Chapter 4) but Nrg1/ErbB have also been implicated in synapse formation and recent research has focus on the role of this signalling pathway in wiring of the cerebral cortex.

Nrg1 type I induces the synthesis of acetylcholine receptor (AChR) (Falls *et al.,* 1993). Nrg1-controlled synthesis of AChR decreases with inhibition of ErbB activation and Erk, JNK and CDK5 kinases (reviewed by Mei and Xiong, 2008). Contrasting *in vivo* results suggest that Nrg1/ErbB signalling pathway has a redundant effect in the postsynaptic development. Nrg1 happloinsufficient mice show a reduced AChR density (Sandrock *et al.,* 1997), while conditional ablation of ErbB2 and ErbB4 showed a normal development of neuromuscular junction (Escher *et al.*, 2005).
Schizophrenia has been associated with anomalous synaptic plasticity and neurotransmission. Both these processes have been associated with Nrg1 in the adult brain. Neuregulin 1 type III regulates expression of AChR while neuregulin 1 type I and II have been associated with modifications of the GABA receptors (Stefansson *et al.,* 2002). All three isoforms are capable of regulating NMDA and AMPA receptors. ErbB4 signalling was also shown to regulate excitatory synapses and disruption of Nrg1/ErbB4 signalling affects the AMPA receptors, leading to loss of NMDA receptors (Li *et al.,* 2007).

Neuropathological features

Neuropathological features associated with schizophrenia mainly comprise macroscopic and histological changes such as enlargement of the ventricles, abnormal cortical thickness, reduced cerebral volume, morphological and molecular changes in the hippocampus, dorsal thalamus and dorsolateral prefrontal cortex

characterized by atypical clusters of neurons, as well as their aberrant location, and cerebral asymmetry (Harrison and Weinberger, 2005). Although all of these characteristics are subtle, they are not present across all cases of schizophrenia.

Clinical studies combined with neuroimaging and post-mortem studies have identified the superior temporal gyrus (STG) and prefrontal cortex (PFC) as the main brain structures implicated in schizophrenia. These specific brain structures may help elucidate some of the main symptoms related to schizophrenia. The STG controls speech, auditory processing and social cognition and changes in this brain structure may be linked with the common positive symptoms of schizophrenia, such as delusions, disordered thoughts and speech and different types of hallucination (ie, auditory, visual and olfactory). The normal function of the PFC comprises complex cognitive behaviour, decision making and restraining social behaviour. In schizophrenic patients it is thought that the PFC dysfunction is linked with cognitive disturbances and abnormalities of synaptic structure and function in this cortical region (Keshavan *et al*., 1998; Shapleske *et al*., 1999).

Loss of grey matter in Schizophrenia

Using magnetic resonance imaging (MRI) it has been showed that during normal human development the grey matter volume is spatiotemporally altered (Gogtay and Thompson, 2010). Cortical development is characterized by a dynamic rate of maturation of the distinct lobes of the cortex, with lower-order somatosensory and visual centre maturation preceding higher order association cortices (i.e., language related areas) and maturation of grey matter (Gogtay *et al.*, 2004). During development the brain goes through a re-wiring process which involves the loss of cortical grey matter and a decrease of synapse density in order to maximize the efficacy of transmission of synapses (Bennet, 1999; Huttenlocher and Dabholkar, 1997). In studies of patients with schizophrenia, MRI investigations show a decrease in grey matter volume in the prefrontal temporal network of the brain specifically in the insula cortex, the superior temporal gyrus, medial temporal lobe, medial and inferior frontal gyrus and the anterior cingulate gyrus (reviewed by Bennet, 2011). Loss of grey matter during normal development is completed around 22 years of age,

when MRI from brains of patients with schizophrenia are compared with healthy individuals this loss is accentuated by 10 percent. A possible explanation for a greater loss of grey matter in these patients is the potential depletion of synapses.

Loss of white matter in Schizophrenia

White matter abnormalities are a common feature of schizophrenia and bipolar disease. Research using MRI has detected a decrease in white matter density in the anterior limb of the internal capsule and in the prefrontal subgyral white matter (McIntosh *et al.,* 2005; Zhou *et al.,* 2003). Interestingly, these studies provided evidence for a genetic background since this phenotype is shared by the control individuals (unaffected relatives of patients) (McIntosh *et al*., 2006). Studies performed in post mortem-tissue have identified high levels of expression of Nrg1 type IV, specifically the SNP rs6994992 (SNP8NRG243177) that affects white matter integrity and density (Law *et al.,* 2006; McIntosh *et al.,* 2008; Sprooten *et al.,* 2009).

Involvement of myelination in schizophrenia

The pathogenesis of schizophrenia is associated with different areas of the brain, their respective connections and morphological change. Although the etiology of schizophrenia remains elusive, and the primary concept of schizophrenia being a neurodegenerative disorder (now know to be mainly a neurodevelopmental disease) has led researchers to investigate the role played by myelin and the myelinating cells of the brain – oligodendrocytes.

Although this is a recent topic of research there is some evidence that myelination plays a role in schizophrenia. The loss of white matter in patients with schizophrenia is attributed to myelination defects throughout development. In these patients there is a decreased volume, compromised integrity and abnormal growth of white matter which translates into cognitive and sensorimotor coordination deficits (Chew *et al*., 2013). Areas such as the corpus callosum, frontal lobe and temporal lobe are linked

with the neuropathological traits of schizophrenia and are associated with myelin aberrations and oligodendrocyte degeneration. Hakak and co-workers (2012) shed some light into the dysregulation associated with myelination in schizophrenia with a genome-wide expression analysis. After analysing the dorsolateral prefrontal cortex of schizophrenic patients and matching controls the study identified various genes related to myelination, and also other biological processes such as synaptic plasticity, neurotransmission, neuronal development and signal transduction (Hakak *et al*., 2012). Interestingly, this study identified four genes specifically related to myelination and oligodendrocyte development (myelin and lymphocyte protein - MAL, myelin associated glycoprotein - MAG, 2',3'-cyclic nucleotide 3'-phosphodiesterase – CNPase and transferrin). All of these genes were down regulated in the post-mortem brains of schizophrenics implying a failure of oligodendrocyte function (Hakak *et al.,* 2012). Another recent study expanded the search for glial genetic links in schizophrenic patients (Goudriaan *et al.,* 2013). Oligodendrocytes have been implicated in the pathophysiology of schizophrenia due to the decreased white matter volume, but there are new lines of evidence showing that astrocytes and microglia also contribute to the pathophysiology of schizophrenia via neuroinflammatory processes (Goudriaan *et al.,* 2013).

Myelination alterations have also been shown in the phencyclidine (PCP)-induced neurodevelopmental animal model of schizophrenia. The pharmacological animal model of PCP administration results in prepulse inhibition (PPI) deficits and hyperlocomotor activity in rats, which have both been extensively described in schizophrenic subjects. Progenitor oligodendrocytes express NMDA receptors, PCP is an NMDA receptor antagonist and after administration of PCP in rats at postnatal day 2, animals were subjected to behavioural tests (locomotion and PPI) and subsequently, analysed for myelin aberrations and oligodendrocyte maturation. Myelin was quantified by MBP expression at postnatal days 16, 22 and 32, showing a decreased MBP expression in the frontal cortex. Oligodendrocyte maturation was analysed by glutathione S-transferase-π (a marker of oligodendrocyte maturation), showing a loss of mature oligodendrocytes also in the frontal cortex (Zhang *et*

al.2012). Together, these results show a correlation between the behavioural deficits present in schizophrenia with the abnormalities observed in the white matter.

5.1.5 Animal models of schizophrenia

Schizophrenia is a complex disorder presenting a constellation of positive, negative and cognitive deficit symptoms. Due to the range of the many symptoms presented by different patients and the non-existence of a single risk-factor modelling schizophrenia remains one of the most difficult tasks in the psychiatric field. Therefore the field has evolved to generate models that mimic specific functions instead of the entire array of symptoms. These animal models need to be able to characterize genetic, neural and neurochemical features that can be correlated with the human pathology. In the case of schizophrenia the negative symptoms are shared between depression and autism, consequently models for these disorders are also useful to partially understand schizophrenia. Thus far, different types of approaches have been made to generate such models which involve a) pharmacological or drug-induced models, involving the manipulation of different neurotransmitters systems (e.g. Amphetamine: dopamine indirect agonist and phencyclidine/ketamine: NMDA receptor antagonist); b) lesion models that modulate the neurodegeneration (e.g. neonatal ventral hippocampal lesion); c) development models that allow neurodevelopment characterization (e.g. gestational X-irradiation); and d) genetic models associated with the genetic candidates as risk factors (e.g. neuregulin and DISC1 disruption) (Fernando and Robbins, 2011).

Modelling positive symptoms associated with schizophrenia such as delusions and hallucinations is far more complex since the diagnosis of these features in human patients are based on verbal description; therefore it is necessary to overcome such issues in animal models. It has been reported that rhesus monkeys treated subchronically with amphetamine exhibit signs of hallucinations (Nielsen *et al.*, 1983). Cognitive disturbances associated with schizophrenia such as working memory, planning, cognitive flexibility and control can be measured in animals (Hagan and Jones, 2005; Robbins and Moore, 2008). The cognitive deficits observed

in this disorder correlate with the anatomical features observed in patients, where there is a loss of grey and white matter (Robbins and Moore, 2008). Currently, research is being focused in genetic models disturbing different signalling pathways that may lead to decreased density of white and grey matter, such as the Nrg1. Models to converge both the behavioural and anatomic relation and its association with genetic and environmental factors have been proposed over the years. One of these models is the dominant-negative mice for the human DISC1 that were embryonically injected with polyriboinosinic-polyribocytidilic acid (polyI : C) which promotes a time-limited production of maternal pro-inflammatory cytokines in uterus. During adulthood these mice show signs of behavioural, cognitive and pharmacological abnormalities, being the prime example for modulation of genetic and environmental interactions (Nagai *et al.*, 2011).

5.2 Results

According to several studies, schizophrenia patients show decreased cerebral (cortical and hippocampal) volume (reviewed by Harrison, 1999). Several structural changes have been observed such as ventricle enlargement, reduction of frontal and temporal lobe volume as well as cerebellum (Schroder *et al*., 2002). Changes in the hippocampus have also been described in schizophrenia, with hippocampal volume reduction being a prominent feature in the brain of these patients, thought to contribute to the deficits in working memory observed in these individuals (Heckers, 2001). These brain alterations have been linked to myelination, with different explanations arising, such as a decrease in axonal number or diameter, an overall reduction of myelination, or oligodendroglial dysfunction (Chew *et al*., 2013; Palaniyappan *et al*., 2013). Neuregulin 1 has been identified as a genetic risk factor for schizophrenia, and since it has been demonstrated that these patients have increased levels of neuregulin 1 type IV in a multi-collaboration project we set out to investigate and characterize myelination in mice overexpressing neuregulin 1 type IV, in order to assess if neuregulin 1 type IV regulates any of the morphological aspects that have been described in schizophrenic individuals.

To investigate the role of neuregulin 1 type IV in the central nervous system we analysed the morphological structure of the brain by immunohistochemistry using haematoxylin and eosin staining and luxol fast blue. Mice were perfused with 4% paraformaldehyde in 0.1M phosphate buffer and post fixed overnight. Brains were dissected and snap freezed for cryosectioning. Immunohistochemistry was performed after these brains were scanned for MRI by Dr. Andrew McIntosh. Haematoxylin stains for cells nuclei in a deep-blue colour and eosin stains proteins nonspecifically and has a pink colour. Analysing sagittal sections of the cerebellum and coronal sections of the hippocampus showed no structural differences between wildtype and overexpressing neuregulin 1 type IV mice (Fig. 5.1 – 5.4). To test whether these mice had any alterations in myelination we also performed a luxol fast blue staining. Luxol fast blue is the alcohol soluble counterpart of the water soluble alsian blue and stains for myelin lipoproteins. Analysis of sagittal and coronal sections showed no myelin abnormality in the corpus callosum, cerebellum and hippocampus of overexpressing neuregulin 1 type IV mice when compared with their wildtype counter parts (Fig. 5.5 – 5.8).

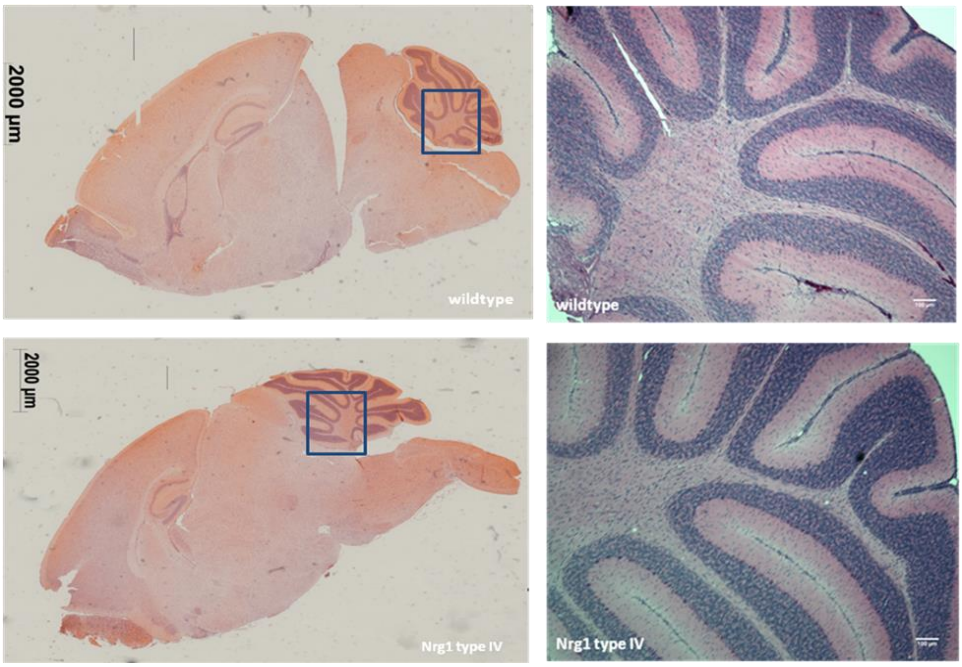


Figure 5.1 – Normal morphology of cerebellum in mice overexpressing neuregulin 1 type IV at postnatal day 17 (P17). Haematoxylin and eosin staining cerebellum sagittal sections of wildtype and Nrg1 type IV overexpressing mice at P17 (4x magnification, scale bar: 100µm).

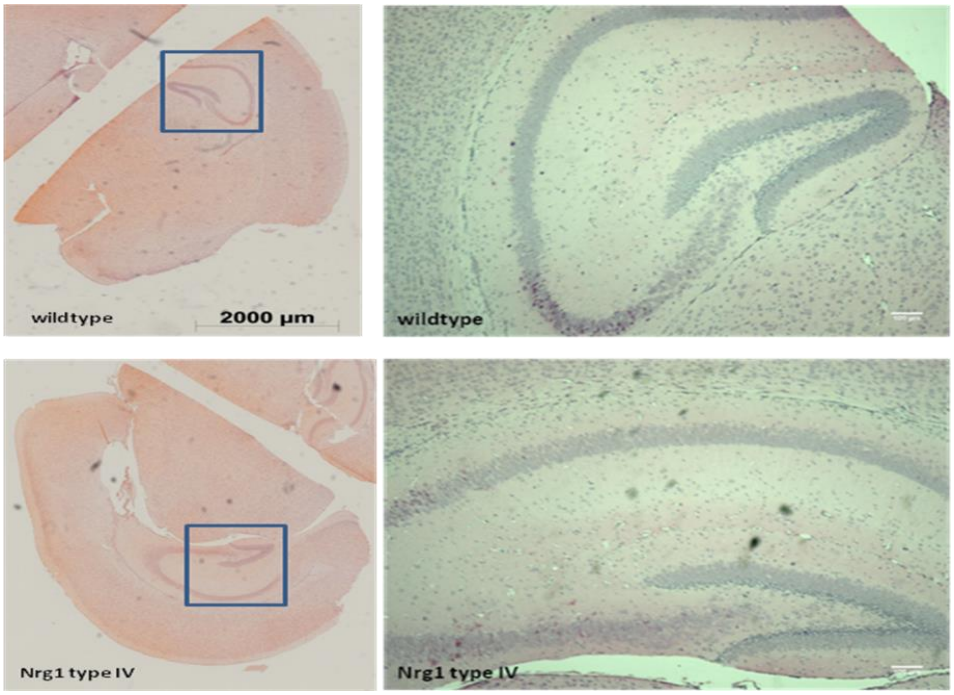


Figure 5.2 – Overexpression of neuregulin 1 type IV does not alter the morphology of hippocampus (P17). Coronal sections of hippocampus with haematoxylin and eosin staining of wildtype and neuregulin 1 type IV overexpressing mice at P17 (4x magnification, scale bar: 100µm).

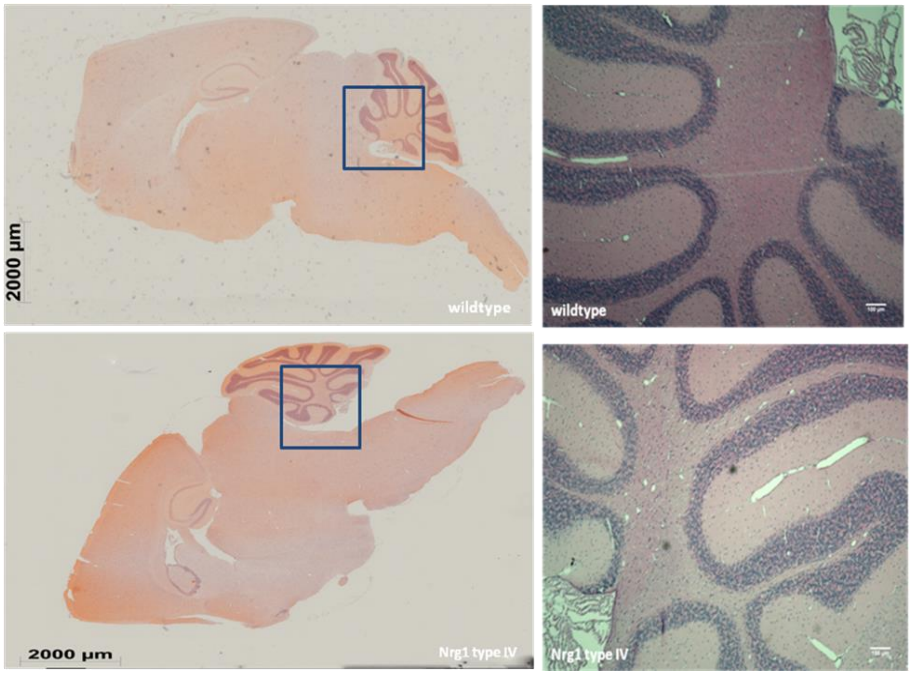


Figure 5.3 – Overexpression of neuregulin 1 type IV shows normal morphology of cerebellum in older mice. Sagittal sections of cerebellum stained for haematoxylin and

eosin of wildtype and neuregulin 1 type IV overexpressing mice at postnatal day 60 (P60) (4x magnification, scale bar: 100μm).

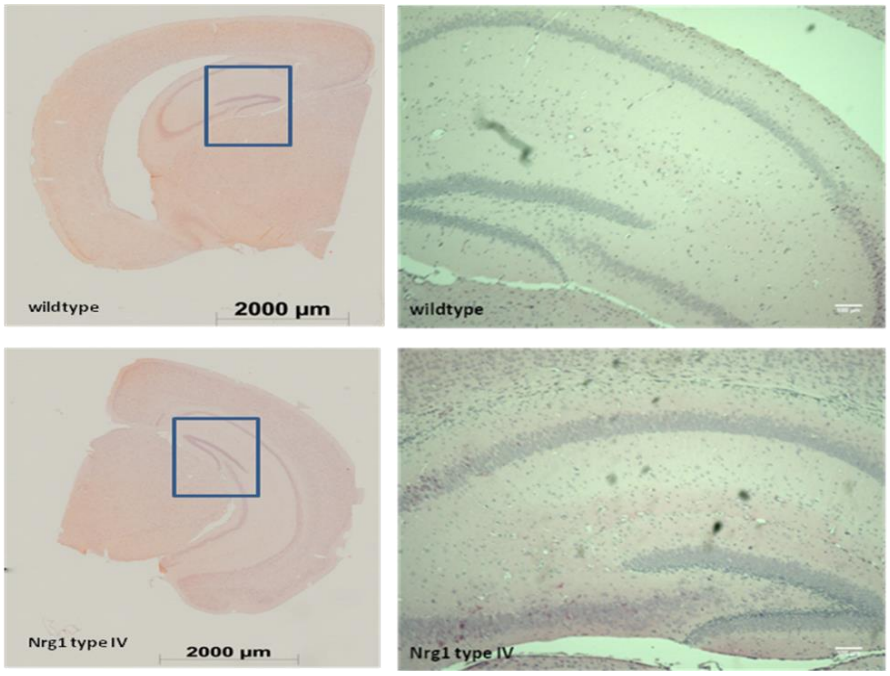


Figure 5.4 – Normal morphology of hippocampus with overexpression of neuregulin 1 type IV in mice at postnatal day 60. Coronal sections of hippocampus with haematoxylin and eosin staining of wildtype and neuregulin 1 type IV overexpressing mice at P60 (4x magnification, scale bar: 100μm).

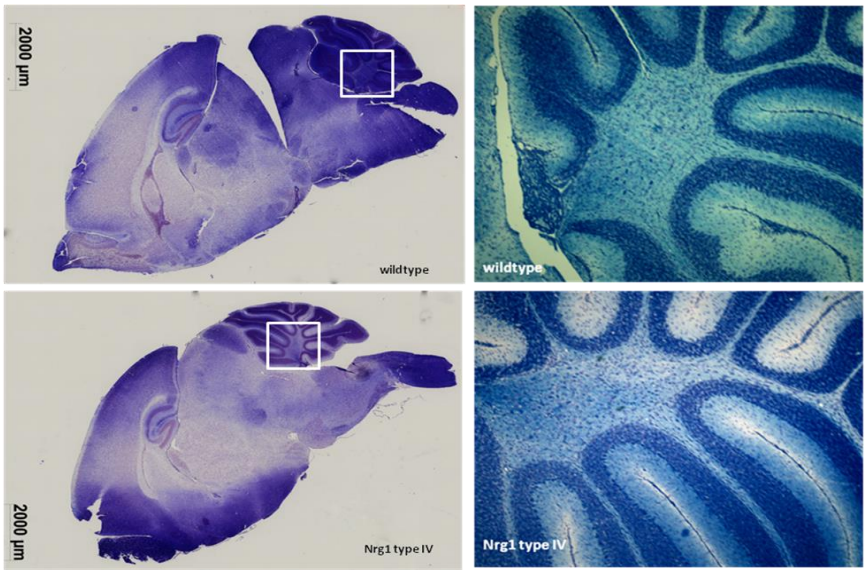


Figure 5.5 – Myelin morphology is unaffected by overexpression of neuregulin 1 type IV. Sagittal sections of cerebellum stained for luxol fast blue of wildtype and neuregulin 1 type IV overexpressing mice at postnatal day 17 (P17) (4x magnification, scale bar: 100μm).

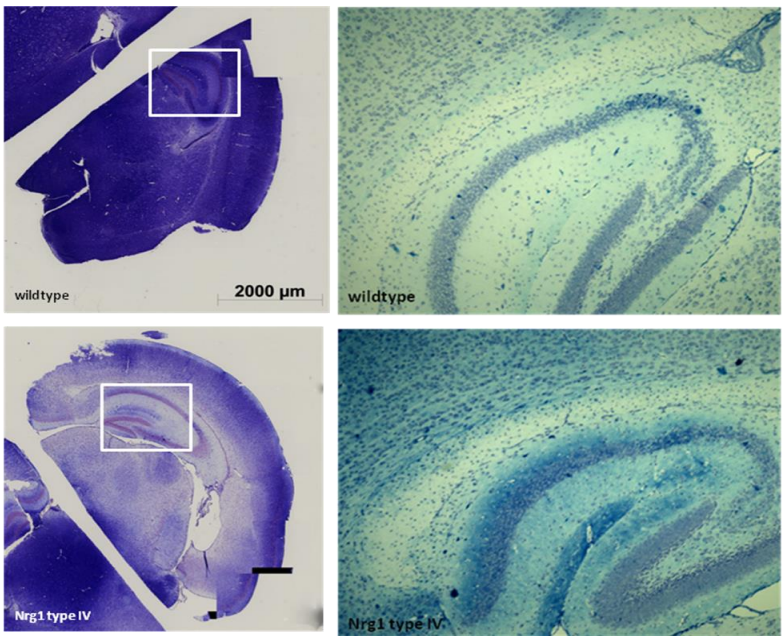


Figure 5.6 – Unaltered myelin morphology of hippocampus and corpus callosum of overexpressing neuregulin 1 type IV mice. Sagittal sections of hippocampus stained for luxol fast blue of wildtype and neuregulin 1 type IV overexpressing mice at postnatal day 17 (P17) (4x magnification, scale bar: 100µm).

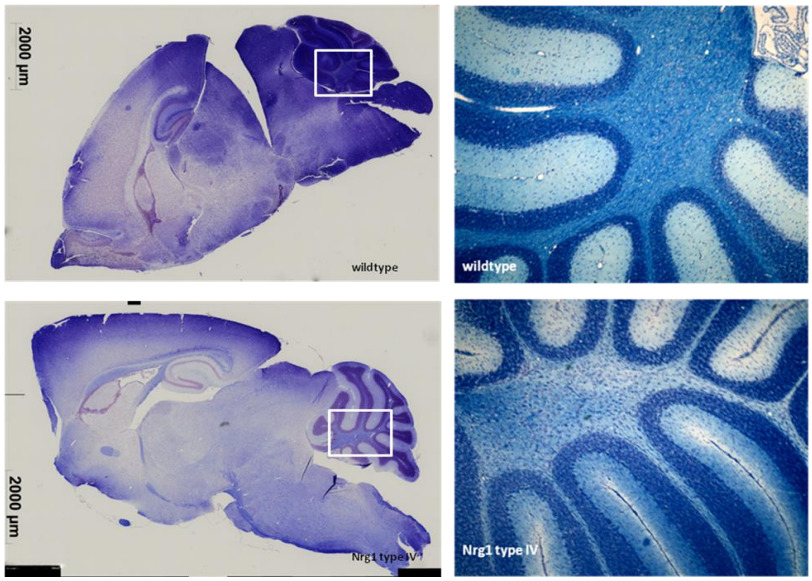


Figure 5.7 - Overexpression of neuregulin 1 type IV shows normal myelination of the cerebellum in older mice. Sagittal sections of cerebellum stained for luxol fast blue for wildtype and neuregulin 1 type IV overexpressing mice at postnatal day 60 (P60) (4x magnification, scale bar: 100µm).

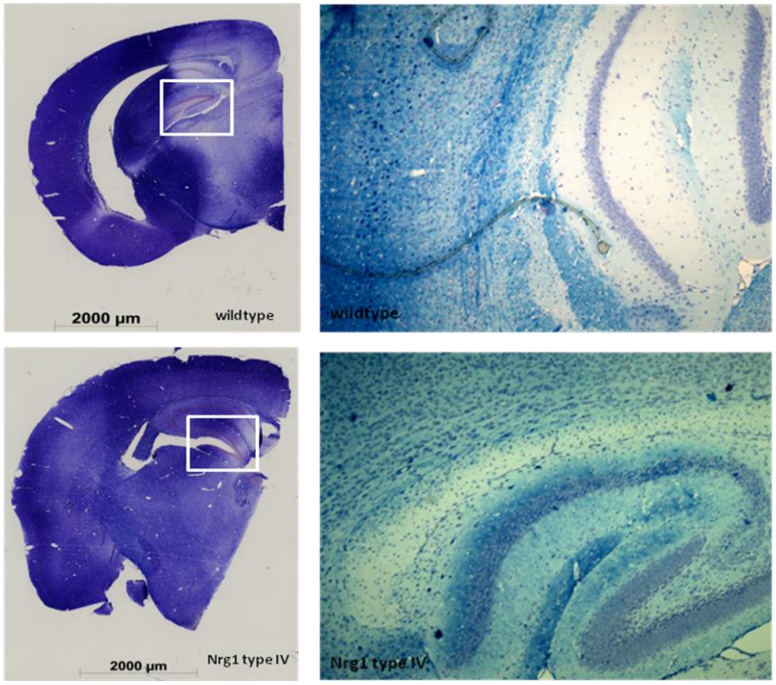
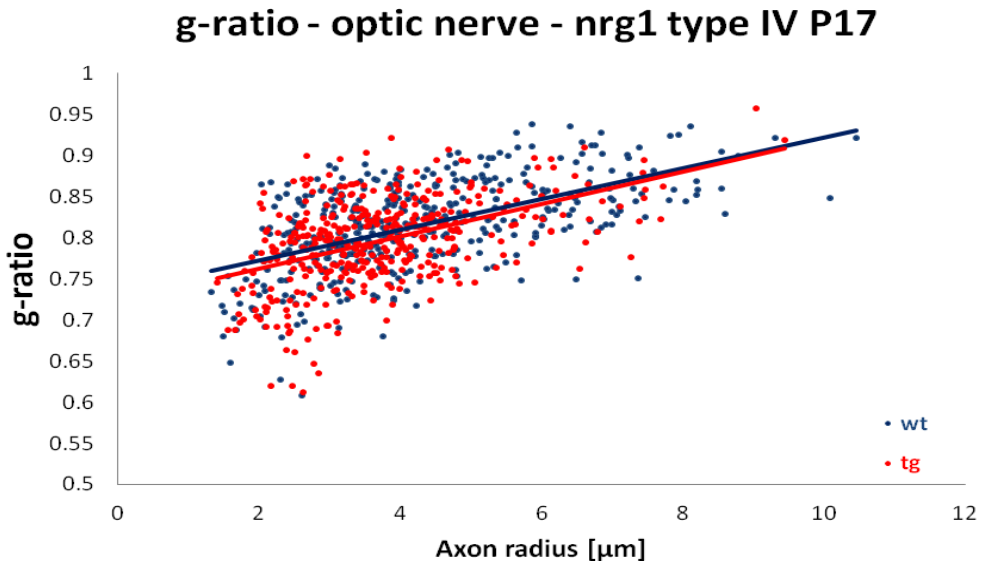


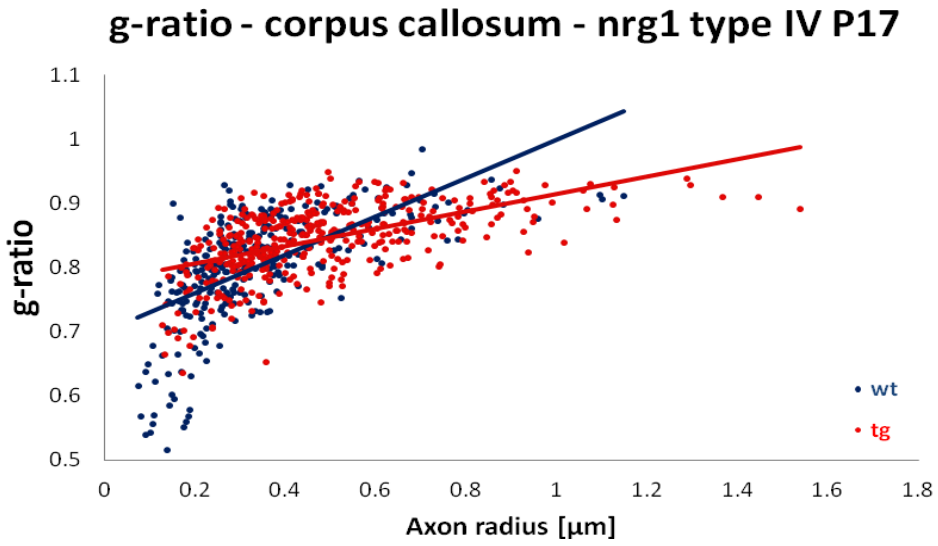
Figure 5.8 – Normal myelination of hippocampus and corpus callosum of neuregulin 1 type IV mice at postnatal day 60 (P60). . Coronal sections of hippocampus with luxol fast blue staining of wildtype and neuregulin 1 type IV overexpressing mice at P60 (4x magnification, scale bar: 100µm).

An analysis of immunohistochemistry images is complex since most of it is based on intensity of staining, which can be affected by the exposure of the tissue to the staining agent. In this project we focused on qualitative measures to assess integrity of structures, distribution and localization of staining and myelin abnormalities. All structures analysed (corpus callosum and hippocampus) showed structure integrity with no noticeable qualitative change of size, shape or volume. Distribution and localization of staining showed no qualitative abnormalities of myelin thickness or myelin track integrity. Throughout all sections staining was uniform with no patchy staining, no signs of cysts or myelin aberrations, or myelin outside the tracts. Complementary data (not shown) from MRI performed in our collaborator's laboratory confirmed that no abnormalities were seen in the neuregulin 1 type IV overexpressing mice when compared to wildtype.

To further test if overexpression of neuregulin 1 type IV in mice would affect white matter myelination and structure we analysed the optic nerve and corpus callosum of these animals at postnatal day 17 (P17), a time point where myelination is not yet

completed and postnatal day 60 (P60) a time point when myelination has reached a stable turnover. Mice were perfused intracardially with a fixative solution of 4% paraformaldehyde and 3% glutaraldehyde in 0.1M phosphate buffer and post-fixed overnight (perfusions were carried on in Dr. Amanda Law's lab, Maryland, USA). Tissue was dissected and processed for electron microscopy and ultra-thin sections (prepared in the Department of Anatomy, University of Cambridge, UK) were used to photograph non-overlapping regions of both optic nerve and corpus callosum. G-ratio measurements were done using Openlab image analysis software (Improvision) with at least 100 axons measured per animal. Early in development, when myelination is not yet completed (P17) we saw no differences (P = 0.23) between the g-ration of wild type mice (average g-ration of 0.81 ± 0.02, n = 404 axons, 4 animals) and neuregulin 1 type IV overexpressing mice (average g-ration of 0.80 ± 0.02, n = 406 axons, 4 animals) in the optic nerve (Fig 5.9). In the corpus callosum, no differences were observed (P = 0.16) when comparing neuregulin 1 type IV overexpressing mice (average of 0.84 ± 0.02, n = 405 axons, 4 animal) to wild type mice (average of 0.80 ± 0.02, n = 302 axons, 3 animals) (Fig 5.9). In the optic nerve at P60 there was no difference between genotypes with wildtype average g-ratio of 0.86 ± 0.01, and neuregulin 1 type IV overexpressing mice 0.84 ± 0.01 (Fig. 5.10). Similarly in the corpus callosum overexpression of neuregulin 1 type IV at P60 (average g-ratio 0.78 ± 0.01, n = 363, 4 animals) did not affect myelin thickness when compared to wildtype (average of 0.76 ± 0.02, n = 405, 4 animals) (P = 0.45) (Fig. 5.10).





5.9 - Unaltered myelin morphology in overexpressing neuregulin 1 type IV mice at postnatal day 17. Upper panel: g-ratio measurements as function of axon radius of optic nerve is not significantly (P=0.23) between wildtype (average of 0.81 ± 0.02, n = 404 axons per 4 animals) and neuregulin 1 type IV overexpressing mice (average of 0.80 ± 0.02, n = 406 axons per 4 animals). Bottom panel: overexpression of neuregulin 1 type IV does not alter myelin integrity in the corpus callosum (average of 0.84 ± 0.02, n = 405 axons per 4 animal) when compared with wild type mice (average of 0.80 ± 0.02, n = 302 axons per 3 animals) (P = 0.16). Statistical analyses performed by Student's t-test.

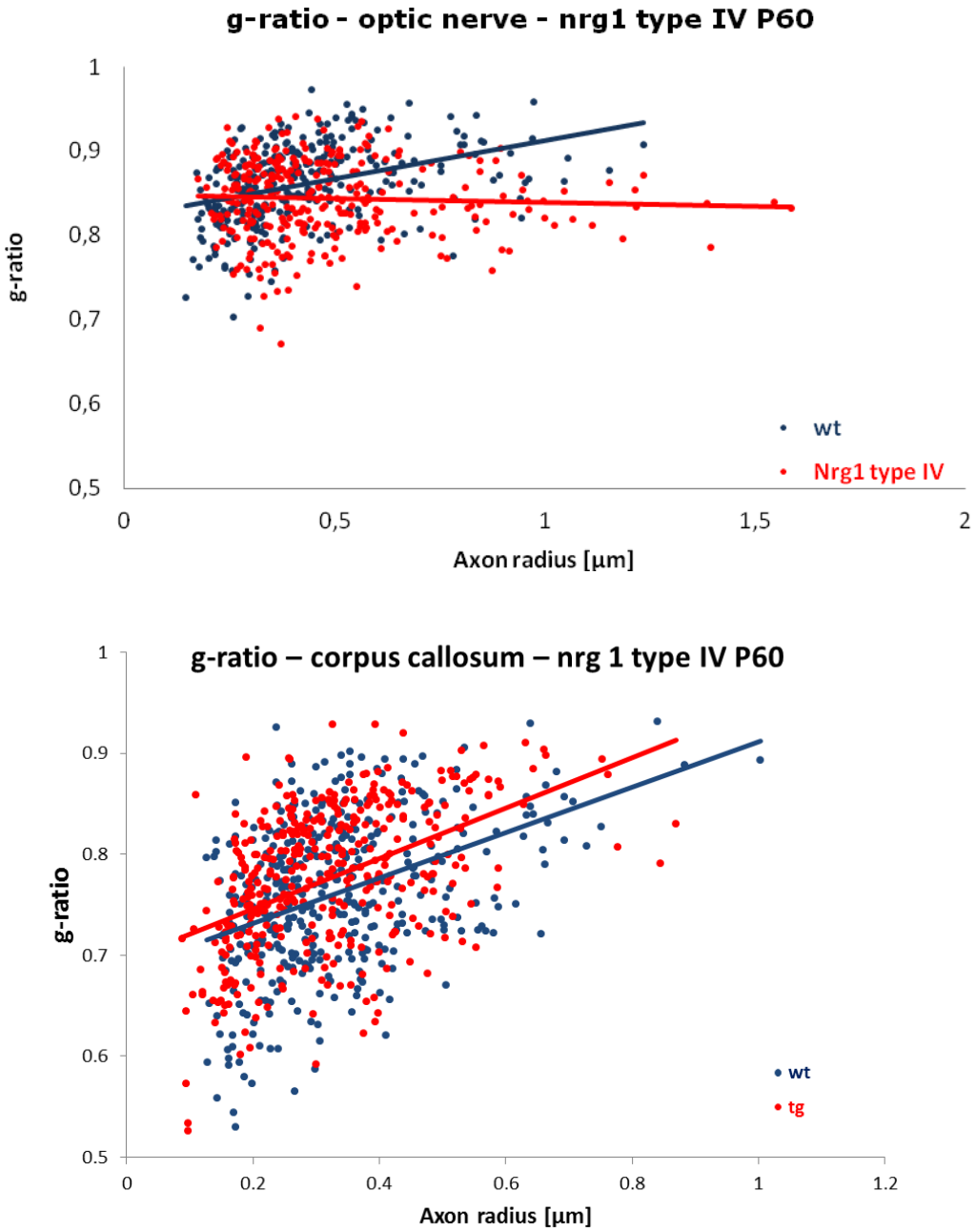


Figure 5.10 – Unaltered myelin morphology in overexpressing neuregulin 1 type IV mice. Upper panel: g-ratio measurements as function of axon radius of optic nerve is not significantly (P=0.31) between wildtype (average of 0.86 ± 0.01, n = 300 axons per 3 animals) and neuregulin 1 type IV overexpressing mice (average of 0.84 ± 0.01, n = 307 axons per 3 animals). Bottom panel: overexpression of neuregulin 1 type IV does not alter myelin integrity in the corpus callosum (average of 0.78 ± 0.01, n = 363 axons per 4 animal) when compared with wildtype mice (average of 0.76 ± 0.02, n = 405 axons per 4 animals) (P = 0.45).

5.3 Discussion

Schizophrenia is a mental disorder that affects 0.5% to 1% of the general population (Stefansson *et al*., 2002). It manifests itself in a wide range of symptoms that are variable between patients. There is no known cause for schizophrenia but recent research has pointed that a combination of genetic and environment factors is the base of this disorder. Typically, schizophrenia comprises a triad of negative, positive and cognitive symptoms. Some of these symptoms can be alleviate with medication, but no cure has yet been found. It is therefore important to understand the mechanisms that underlie this disorder. Different studies have suggested a genetic association between specific genes and different population. One of these genes was neuregulin 1 that was initially reported to be a susceptibility gene after studies in patients in Iceland (Stefansson *et al.*, 2002). Functional studies of neuregulin 1 support the view that neuregulin 1 is involved in the pathogenesis of schizophrenia, with elevated neuregulin 1 and ErbB4 protein detected in the prefrontal cortex of schizophrenic patients (Chong *et al.*, 2008). Two transgenic mice lines support this view, with mice hypomorphic for neuregulin 1 and mice hypomorphic for ErbB4 showing behavioural abnormalities (Gerlai *et al.*, 2000). Interestingly, the neuregulin 1 mice recover after administration of the drug clozapine. These mice also showed a decreased in functional NMDA receptor which is consistent with human studies (Ibrahim *et al.*, 2000).

New technologies such as MRI have been a helpful tool to understand and characterize abnormalities in the brain that could then be linked to specific factors/genes. There is evidence that schizophrenic patients present structural changes in their brains (Arnold and Trojanowski, 1996). In studies of monozygous twins, where only one of the individuals is affect by schizophrenia, there is an enlargement of the lateral ventricles and a decreased cortical area in the affect patient. One possible explanation could be an impairment in neurodevelopment, such as abnormal neural migration and differentiation. This could be controlled by

Nrg1/ErbB signalling since this molecule complex is involved in migration by promoting the formation and differentiation of radial glia.

Hippocampal electrophysiology studies suggest that neuregulin 1 affects long term potentiation (Chen *et al.,* 2010; Huang *et al.,* 2000; Kwon *et al.,* 2005, 2008; Pitcher *et al.,* 2008). Different MRI studies have also shown a loss of hippocampal grey matter in schizophrenic patients (Glahn *et al.,* 2008; Honea *et al.,* 2005; Wright *et al.,* 2000; Nelson *et al.,* 1998; Fornito *et al.,* 2009). It has been suggested that this loss might be due to a combination of decreased numbers of interneurons (Benes *et al.,* 1986; Jeste and Lohr, 1989; Weis *et al.,* 2007) and oligodendrocytes (Chambers and Perrone-Bizzozero, 2004). Other reports have shown a decreased of hippocampal synapses and their dendrites (Rosoklija *et al.,* 2000; Tcherepanov and Sokolov, 1997).

Although in humans myelination is fully complete around two years of age, this process extends throughout adolescence and early adulthood, a time typically associated with the first onset of schizophrenia. Alterations in myelination in this later stage have been observed through histological and imaging studies. Microscopy studies revealed white matter alterations in schizophrenia, such as abnormal myelin sheath lamellae, decreased mitochondrial volume in oligodendrocytes, and oligodendrocyte apoptosis and necrosis in the prefrontal cortex (Uranova *et al.,* 2001). In the prefrontal cortex, it was also shown by microarray data a decreased in oligodendrocyte-related mRNA (Hakak *et al.,* 2001). Nrg1/ErbB signalling is known to regulate oligodendrocyte development (reviewed in Chapter 4), and by inducing oligodendrocyte differentiation or promoting survival of progenitor oligodendrocytes, could promote myelination.

Recently, neuregulin 1 type I and type IV overexpressing mice have been developed by modifying the isoform-specific promoter regions (Deakin *et al.,* 2009). These mice have been behaviourally characterized but their white and grey matter phenotypes were not the focus of investigation. In this project we tested if there were white matter abnormalities in the neuregulin 1 type IV overexpressing mice,

specifically if these mice showed a reduced g-ratio in the optic nerve and corpus callosum. In parallel, our collaborator Dr. Andrew McIntosh tested for a decreased in white matter density by performing MRI techniques (diffusion tensor and T_2-weighted imaging) of fixed brains. These mice showed normal myelin morphology and by analysing light microscopic slides of coronal and sagittal section of these brains, no clear abnormality was observed in either white or grey matter. These results are corroborated by our collaborator Dr. Andrew McIntosh which imaged these brains using diffusion tensor and T_2-weighed and saw no evidence of loss of white matter (unpublished data).

The absence of phenotype in the neuregulin 1 type IV overexpressing mice can be explained by either a compensatory mechanism that buffers the high levels of neuregulin 1 (Tan *et al.,* 2007), since neuregulin 1 type IV (and type I) cellular distribution is similar to one observed for neuregulin 2 (Longart *et al.*, 2004). Or it is possible that the interactions in mice do not mimic the human and therefore no phenotypical changes would be observed. Interestingly, molecular and cellular characterization of the human neuregulin 1 type IV isoform shows that when this human brain specific isoform is transfected to rat hippocampal neurons, it is biologically functional (Shamir and Buonanno, 2011). This isoform is restricted to the neuronal soma and dendrites and it is absent from the axonal surface (Shamir and Buonanno, 2011). These studies were performed in rat, so it is still a possibility that mice specificity is lacking in this isoform and does not interact with its partners. It is also possible that due to the neuronal distribution of neuregulin 1 type IV not being concentrated in the axon, no effect would be detected in myelination but possibly in synapses, so further studies should focus on electrophysiology since it is a highly significant area for exploratory studies, with imbalance of synapses possibly leading to schizophrenia (Terauchi *et al.,* 2010). It would be an important step for the full characterization of these mice to understand the full spectrum of overexpression of neuregulin type IV, not only through behavioural studies but also a complete set of molecular profiling.

A careful analysis of obtained data shows no major alteration in morphology or myelination pattern. It would be interesting to investigate if these mice show any specific phenotype associated with oligodendrocytes. One of the possible ways to access the impact in oligodendrocyte development would be *in vitro*, by using a co-culture system and confirm if there were any alterations in myelination, number of oligodendrocyte processes or internodal length. Due to the expression pattern of the neuregulin 1 type IV, it is possible that this isoform targets glial cells (Shamir and Buonanno, 2011).

Our work shows that even after identification of a susceptibility gene it is difficult to attribute a specific schizophrenic symptom with a specific mutation. Several genes have been proposed to affect schizophrenic patients, but the environmental factors might have a stronger impact in the course of the disease than expected. As prospective future work, a comorbidity animal model should be used, with the genetic factor (overexpression of neuregulin 1 type IV) and an environmental factor such as maternal infection, in order to assess the magnitude of the combined effect and contribution of both factors. In light of the recent study published by Makinodan and colleagues (2012), where the existence of a critical period for social isolation (two weeks after weaning) leads to disruption of prefrontal cortex function and myelination impairments (with reduced levels of the neuregulin 1 receptor ErbB3), it would be interesting to reproduce this experiments with the overexpressing neuregulin 1 type IV mice. If juvenile social isolation leads to a reduction of ErbB receptor, that in turn results in myelination abnormalities, we could hypothesise that these changes would be enhanced in the overexpressing neuregulin 1 type IV mice, explaining why we did not see a myelinating phenotype in these mice, because the alterations would be dependent on social cues.

Schizophrenia is a debilitating disease with no effective treatment. Diagnose is based on patients' profiling according to different symptoms and the durations of those symptoms. Since some of the symptomatology of schizophrenia is shared with other psychiatric disorders (such as bipolar disorder) it may be the case that what the scientific and medical community describe as schizophrenia is actually a series of

psychiatric diseases within a schizophrenic spectrum. It is therefore important to shed light into the etiology of schizophrenia as a whole, potentially looking for a biomarker and focus on the integration of multiple hypothesis of etiology, with special focus on how neurotransmitters and myelination affect each other, and the interactions between those and genetic and environmental factors.

Chapter 6

Conclusions and Perspectives

Chapter 6

Conclusions and Perspectives

Myelination is a tightly controlled process where a glial cell wraps several layers of myelin around an axon in order to insulate it, improve conduction performance, and provide long term integrity of the axons (Griffiths *et al.*, 1998; Huxley and Stampfli, 1949). Central nervous system (CNS) and peripheral nervous system (PNS) myelination differ in terms of the cells responsible for myelination (oligodendrocytes and Schwann cells, respectively) and some of the core proteins that constitute the myelin layer (PLP in the CNS vs P0 in the PNS). Oligodendrocytes can extend several processes and myelinate different axons, while Schwann cells can only myelinate one internode. For myelination to occur a glial cell needs to establish contact with an axon and identify the number of wraps that will provide complete insulation of the axon. The thickness of the myelin sheath is regulated by the axon diameter (Friede, 1972) with an axonal threshold of 0.2 µm in CNS and 1 µm for the PNS (Waxman and Bennet, 1972; Voyvodic, 1989) necessary for myelination to occur. The existence of a threshold for myelination together with the observation that *in vitro* oligodendrocytes are able to extend processes in the absence of neurons, but can only form compact myelin layers in the presence of axons, shows that axonal signalling and/or secreted molecules are necessary for initiation of myelination (Lubetski *et al.*, 1993). Further, transplantation of oligodendrocytes from the optic nerve (an area characterized by small calibre axons) into the spinal cord (with typically large diameter axons) myelinate the correct amount of myelin sheaths showing that myelin thickness is not an intrinsic property of the oligodendrocyte, but regulated by the axon (Fanarraga *et al.*, 1998).

Myelination in the CNS is a very intricate process and the major players that regulate this process have yet to be identified. Central myelination is performed by oligodendrocytes which after development acquire the capacity of myelinating multiple axons. Different approaches to study oligodendrocyte development and key players during myelination have been made throughout the years. In 1980, McCarthy

and Vellis described the first protocol for isolation of relatively pure oligodendrocytes (McCarthy and Vellis, 1980). This was a major step towards understanding the myelination process since it was now possible to study oligodendrocyte development *in vitro*. New advances were made with the co-culture system that has been established as an *in vitro* model to study myelination. In these cultures it is possible to culture axons and glial cells, and track the developmental process of axon ensheathment (Lubetzki *et al*., 1993). Although these models allowed major breakthroughs in the field, new and more complex systems are needed to fully understand myelination, taking into account not only axoglial interaction but also the full environment that can influence myelination. *Ex-vivo* models have also been described, where slices of tissue are maintained in culture permitting a better overview of myelination, since these cultures provide cell-cell and cell-ECM interaction. All of these techniques are crucial for myelination research, but an *in vivo* approach is necessary to fully understand the complexity surrounding myelination, such as transgenic animals. Mutant models provide insight into the molecules regulating different aspects of myelination and associated diseases.

In this thesis I have asked the question of whether integrins play a role in the regulation of myelination, either by contributing to the signals that initiate myelination or by regulating the myelin sheath thickness. I found that mice expressing a dominant negative β1 integrin (that reduces β1 integrin signalling independently of ligand binding) in myelinating oligodendrocytes require a larger axon diameter to initiate myelination. However, this phenotype is transient, with older mice not showing the shift present in younger mice; and large axons are myelinated normally (Câmara *et al.,* 2009). These results suggest that there are other signals in the axon that also contribute to initiation of myelination. Previous studies described neuregulin 1 type III as a regulator of PNS myelination, controlling the decision to myelinate and myelin thickness, although the role of neuregulin 1 type III in the CNS is controversial (Fig. 4.5).

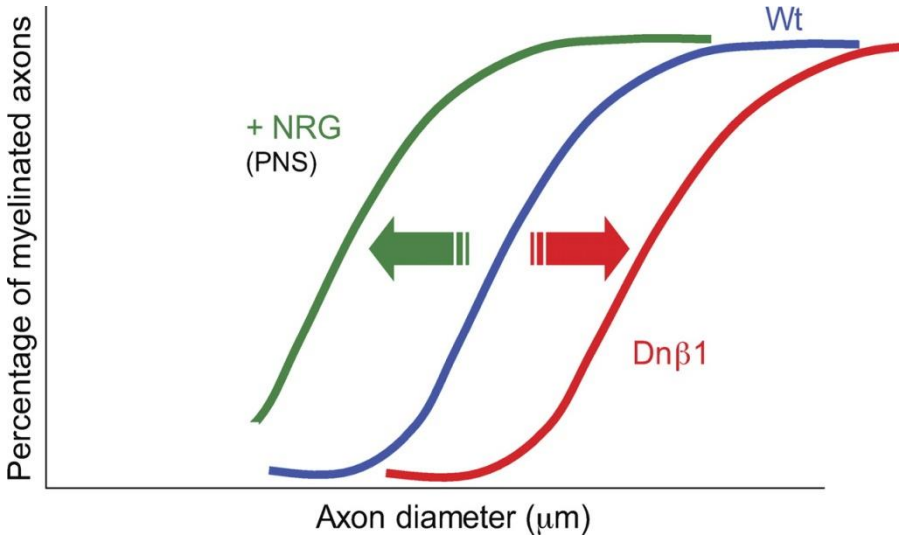


Figure 6.1 – Contrasting effect of neuregulin 1 in the peripheral nervous system and β1 integrin in the central nervous system. In the PNS, overexpression of neuregulin 1 results in a shift to the left of the dose-response curve representing the percentage of myelinated axons plotted against the axon diameter. In the CNS, β1 integrin has the opposite effect with dominant negative β1 integrin mice showing a shift to the right of the diameter threshold for myelination. Reproduced from Câmara *et al.,* 2009.

Since this phenotype is transient it would be interesting to investigate if a delay of the activation of the dominant negative β1 integrin (in these mice under the control of MBP) would result in the same phenotype, or if there is a timely window when β1 integrin is necessary for the correct myelination of small diameter axons. It would also be interesting to repeat this same approach with different integrins that are normally expressed in myelinating oligodendrocytes or even a combination of dominant negative β integrins to assess if different integrins cooperate in the initial axoglial contact and myelination, and possibly in a timely matter in the same pattern that they are expressed biologically to understand if there are any compensatory mechanisms to integrin signalling. To complement these results, a battery of *in vitro* tests could also be performed, such as mixed species cocultures (with either rat neurons and mice oligodendrocytes, or mice neurons and rat oligodendrocytes) with animal specific blocking of β1 integrin, to determine the role of β1 integrin in both neuros and oligodendrocytes.

My second major objective was to establish if oligodendrocyte integrins act in concert with neuregulin 1 expressed in the axons surface to form a recognition complex for axonal signals that determine whether or not myelination occurs. My hypothesis was that in the CNS there are different signalling pathways that contribute to the precise relationship between axon and oligodendrocyte, explaining the lack of phenotype in the neuregulin 1 null mice (Brickmann *et al.,* 2008). This would be consistent with a model where in the CNS ErbB receptors in the oligodendrocyte recognize the axonal neuregulins, which by integrin amplification would generate a signal to initiate myelination. This hypothesis of a multi-molecular signalling complex could explain the transient effect observed in the dominant negative β1 integrin, where older animals seem to recover from the myelination delay seen in younger animals, with a different signalling pathway possibly being involved as a compensatory system that later on restores the normal axoglial interactions.

Initiation of myelination is a crucial step highlighted by studies of post-mortem brains of MS patients (Chang *et al.,* 2002). During early stages of MS, demyelinating lesions can repair effectively through remyelination achieved by newly formed oligodendrocytes. Interestingly, these remyelinating regions show thinner myelin thickness than the one previously achieved during normal development, with progression of MS the remyelination process fails and leads to chronic demyelination and consequently to axon degeneration. Since oligodendrocyte numbers in these chronic lesions are similar to the ones observed in normal development, it is thought that this subsequent inability to remyelinate is due to axoglial contact impairment (Chang *et al.,* 2002).

Studies in the PNS have identified neuregulin 1 type III as a sufficient and necessary signal that controls myelin thickness and the decision for Schwann cell myelination. In contrast to the PNS, in the CNS one such single factor that controls myelination has not been described. This might be due to differences in the process of myelination in the PNS and CNS.

In the PNS, myelination begins with radial sorting, where Schwann cells interact with a bundle of unmyelinated axons and establish a 1:1 relationship with the

correspondent axon to myelinate. During this process β1 integrin and focal adhesion kinase (FAK, a downstream signalling molecule of β1 integrin) are required (Grove *et al.,* 2007; Nodari *et al.,* 2007). Subsequent interaction between axonal neuregulin 1 type III and ErbB2 and ErbB3 receptors expressed in the Schwann cells control the myelination process. As it has been previously described, in the CNS neuregulin 1 type III ablation, and also lack of ErbB3 and ErbB4 has no effect in myelination (Brickmann *et al.,* 2008). Furthermore, oligodendrocytes expressing constitutively active Akt (part of the downstream PI3K signalling pathway) show hypermyelination, contrasting with the PNS where no phenotype was observed (Flores *et al.,* 2008).

Together, these studies highlight the major differences between CNS and PNS myelination. This is likely due to biological differences between the specific glial cells of each system. While Schwann cells align themselves along the axon it will eventually myelinate during the initial stage of myelination, oligodendrocytes need to extend several processes and contact multiple axons before myelination. These means that Schwann cells only have to form a single myelin sheath, while oligodendrocytes will have to form multiple sheaths. This would probably lead to differences in transcriptional mechanisms, with oligodendrocytes needing additional post-transcriptional mechanisms to regulate its different processes. This would emphasize the need for parallel pathways in the CNS responsible for coordination of regulation of myelination, therefore explaining the lack of phenotype observed in the neuregulin 1 knockout, which would be due to compensatory mechanisms. The existence of parallel pathways would mean that in the oligodendrocytes several molecules act in concert to regulate myelination. The identification of such molecules, or complexes of molecules, would prove invaluable to stimulate myelination and remyelination and be targeted for MS therapies.

Since the work described in this thesis shows that β1 integrin and neuregulin 1 type III do not act in concert to control myelination, a possible candidate for regulation of myelination would be contactin. β1 integrin has been shown to control the initial axoglial contact in the CNS and provide a threshold for myelination (Câmara *et al.,* 2009). In an immunoprecipitation assay for α6 integrin (the α subunit that

heterodimerises with β1 and is expressed in myelinating oligodendrocytes) and a proteomic analysis of associating proteins, contactin was one of the proteins shown to associate with the α6 integrin (Laursen *et al.*, 2009). Contactin is expressed both in axons and oligodendrocytes. Axonally expressed L1 and neurofascin 186 are both ligands for glial contactin 1. Therefore contactin-1 would be an ideal candidate for regulation of initiation of myelination.

Mackinodan and co-workers (2012) reported that mice display a critical period where behavioural and cognitive dysfunction will appear after social isolation. Mice isolated for a period of two weeks after weaning show loss of oligodendrocyte ErbB3 receptors and decreased expression of neuregulin 1 (an ErbB3 ligand), together with behavioural and working memory dysfunction. This phenotype is not transient and if mice are then presented with an enriched environment they do not recover, showing that this critical period is fundamental for normal brain function. It would be interesting to take the same approach with the dominant negative β1 integrin // neuregulin 1 type III mice $^{+/-}$, and assess if an isolating period would lead to the same phenotype or if this phenotype would be enhanced by the dominant negative β1 integrin component of these mice.

Multiple sclerosis has been associated with a higher risk of developing schizophrenia (Benros *et al*., 2011). Interestingly, myelination abnormalities have also been described in the pathophysiology of schizophrenia. Multiple sclerosis and schizophrenia share symptoms that can be attributed to this disruption such as cognitive deficit (e.g. working memory) to an onset of dementia. There are a number of parallels between these two conditions. Not only the presence of myelination abnormalities but a change of brain volume (a trade mark of schizophrenia) has also been observed in patients suffering from relapsing-remitting multiple sclerosis (although, in a transient matter) (Rovaris *et al*., 2001). Linkage between autoimmune diseases and psychosis (schizophrenia is considered a psychotic disorder) has been established, since schizophrenia has been associated with factors that modulate the immune system. These can either be explained by genetic factors shared by both autoimmune disorders and psychosis, or common etiological factors such as infections/inflammation (reviewed by Benros *et al*., 2014).

Neuregulin 1 is also involved with schizophrenia. Although our results did not show a phenotype in the overexpressing neuregulin 1 type IV mice, it is of note that one single family of proteins can regulate distinct pathways and affect functions such as myelination and be implicated in mental disorders. It would be interesting to further analyse these mice *in vitro*. Oligodendroglial cultures could be used to check oligodendrocyte morphology, and myelinating cocultures could be used to observe oligodendrocyte myelination up-close. Another approach would be to perform mixed cocultures with wild type and overexpressing neuregulin 1 type IV cells, to assess if myelination would be compromised at the level of neurons or/and oligodendrocytes. As with the dominant negative β1 integrin // neuregulin 1 type III mice $^{+/-}$, following Makinodan work (Makinodan *et al.,* 2012) and since social isolation is known to be a model of schizophrenia, a period of social isolation could also be imposed to these mice to assess any behavioural and morphological brain changes. Schizophrenia is a multifactorial disorder with different environmental and genetic factors being implicated; inducing in utero inflammation in the overexpressing neuregulin 1 type IV mice would be an interesting comorbidity model of schizophrenia. Further investigations would need to be carried out in order to fully unravel the mysteries behind schizophrenia, with more than just one single genetic factor and a combination of environmental factors that would allow a closer scenario to what is seen in schizophrenic patients.

There are a number of weaknesses in this thesis. In terms of technical deficiencies present in these studies the most evident one are the low number of animals used for each set of experiments, with some of them relying on only one or two animals. This is due to the retrospective way these experiments were performed. The main hypothesis of Chapter 3 was that β1 integrin would affect myelination, and the assumption was made that a phenotype would be more prominent in a context of small diameter axons that are present in structures like the optic nerve. Analyses of the optic nerve confirmed our hypothesis by showing a shift to the right of the response curve of the percentage of myelination, with dominant negative β1 integrin mice showing a higher threshold of axon diameter for myelination. Therefore I decided to examine other structures to ask whether this was a tract specific

phenotype, and analyse the tracts of the same animals in order for an unbiased comparison. Unfortunately at the time of dissection not all tissue was dissected and for some animals it was discarded, leaving some of the data sets incomplete and only included in this thesis as an indication of the trend seen in such structures.

Considering the results in Chapter 3, where a phenotype was only observed in the optic nerve for the dominant negative β1 integrin mice, and given that previous research of the neuregulin 1 type III $^{+/-}$ mice had only showed a phenotype in the corpus callosum, I decided to focus my attention in these two brain structures. In hindsight, different structures of the brain should have been considered and analysed. To overcome this frailty more animals should have been bred and analysed, for a complete overview of the effect of integrins and neuregulins in the different structures of the brain.

These leads to another shortcoming identified in this thesis: the lack of power calculation studies. It would have been advantageous to perform a small systematic review at the beginning of each project of all studies published of the current research of β1 integrin (Chapter 3), neuregulin 1 type III (Chapter 4) and neuregulin 1 type IV (Chapter 5) and use these publications and preliminary studies to calculate the number of samples necessary for effect size of event data. A power calculation study would allow for more robust results and better interpretation of data.

Regarding the outcome measures it is important to note that these are mainly from electron microscopy studies. For an extensive characterization of all transgenic lines used, it would have been interesting to not only focus on anatomical changes but complement these studies with behavioural and electrophysiological data. Research published after the conclusion of this work showed that social isolation can impact neuregulin and its receptor ErbB expression, especially in the prefrontal cortex (Makinodan *et al.*, 2012) and is also associated with altered behaviour and reduced learning. This remarkable work therefore shows how conditioning animals (here by varying social stimulation) can lead to modifications in terms of cognitive functions and myelination. Given this, it would have been interesting not only to analyse the behaviour of all the transgenic lines described in this work, but also vary social

stimulation in these animals and examine a possible exacerbation of the phenotypes associated with each genotype.

Further *in vitro* experiments would also be interesting; by manipulating more than one integrin *in vitro* a better understanding of the interconnection of these proteins would be possible and would be an important set of data to understand the role of integrins in myelination. Additionally, a time-course of integrin blocking in co-cultures (where blocking could be species specific for oligodendrocytes, or axons, using a mix culture of rat and mice cells) would enhance our understanding of integrin modulation of myelination.

In terms of experimental designs some of the flaws identified concern the age points chosen. Instead of only two data points (before and after completion of myelination), that in the case of the dominant negative β1 integrin project showed a transient effect, with the phenotype being present in the earlier time point and absent in the later one, at least two other time points should have been included. An earlier time point, such as P10, when myelination is under way, and a later time point such as P120 or later should have been considered to give insight into the timeline of the effect of integrins and neuregulins in myelination. Furthermore, this project did not take into account the contribution or compensation of other integrins. The fact that we see a transient phenotype might be explained by compensatory mechanisms of the full range of integrins expressed in oligodendrocytes. To address this problem, further integrin transgenic lines should have been engineered, the different mice lines crossed and analysed.

In conclusion, a number of weaknesses have been identified and there is scope to improve this work with a further set of experiments that would characterize all transgenic animals in terms of behavioural, electrophysiological and *in vitro* data. Myelination defects are linked with a myriad of pathologies, such as MS and schizophrenia. Understanding this complex biological process is of keen importance, as the discovery of the molecules responsible for myelin regulation could be used as a drug therapy target and prevent or ameliorate these conditions. Therefore, it is relevant for science that myelination research focus on the axoglial contacts but in a

more integrative way, with genetic and environmental factors being consider in the same model to modulate this diseases and give insight into translational therapies.

Chapter 7

Bibliography

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Bibliography

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